Remarks

The present communication includes a Request for Continued Examination, and responds to the Final Office Action mailed February 14, 2006, and in view of the telephonic interview which included Examiner Salvoza, Supervisory Examiner James Housel, and the undersigned attorney. Applicants' attorney thanks Examiner Salvoza and Supervisory Examiner Housel for the courtesies extended during the telephonic interview on April 18, 2006. It is noted here that no decision was reached on the allowability of any claim during the interview. It is further noted that this RCE is being filed at the suggestion of Mr. Housel during the interview. In addition, during the interview Mr. Housel asked to see disclosures of similar assays that are being developed by other workers. To this end, two recent publications (Gabriel et al. and Salomon et al.) are included herein. These publications are included for information purposes only, and have publication dates later than that of the present application's priority date. These publications do not constitute art that can anticipate or render the present claims obvious.

Claim amendments

Support for the present amendments can be found as follows.

Support for claim 1 as amended can be found at least on claim 1 as originally filed, and as follows. Support for "providing a genetically engineered vertebrate cell comprising a recombinant RNA molecule that comprises an artificial segment of a segmented negative strand RNA virus or the complement thereof, the artificial segment comprising a 5' untranslated region (UTR) of a segment," can be found at least on claim 1 and claim 3 as originally filed; support for "virus suspected of being comprised by the sample" can be found at least on page 4 lines 6-9; support for "thereby indicating the absence, presence, or quantity, respectively, of the virus

suspected of being comprised by the sample" can be found at least on page 4 line 23-page 5 line 3.

Support for claim 3 as amended can be found at least on claim 3 as originally filed, and as follows. Support for "suspected of being comprised by the biological sample" can be found at least on p.23 paragraph [0038], lines 14-16 of the specification.

Support for claim 10 as amended can be found at least on claim 10 as originally filed, and as follows. Support for "a segmented negative strand virus in a biological sample" can be found at least on claim 16 as originally filed. Support for "a cDNA of a 5' untranslated region (UTR)," can be found at least on claim 16 as originally filed. Support for "a cDNA of a 3' UTR, wherein at least one of the 5' UTR and the 3' UTR is a UTR of a segmented negative strand RNA virus" can be found at least on page 12, paragraph [0029], lines 18-21 of the specification. Support for "the virus suspected of being comprised by the sample" can be found at least on p.23 paragraph [0038], lines 13-17 of the specification. Support for "the cell lacks at least one nucleocapsid protein of the virus" can be found at least on page 10, paragraph [0026], lines 7-9 of the specification. Support for "contacting the cell with a biological specimen suspected of comprising the virus" can be found at least on page 21, paragraph [0037], lines 18-20 of the specification. Support for "thereby indicating the absence, presence or quantity, respectively, of the virus suspected of being comprised by the sample" can be found at least on page 22, paragraph [0037], lines 2-4 of the specification.

Support for claim 16 as amended can be found at least on claim 16 as originally filed, and as follows. Support for "being comprised by the biological sample" can be found at least on p.23 paragraph [0038] of the specification, lines 14-16.

Support for new claim 95 can be found at least on page 10, paragraph [0026], lines 7-9 of the specification.

Support for new claim 96 can be found at least on page 10, paragraph [0026], lines 7-9 of the specification and page 29, paragraph [0050], lines 5-7 of the specification.

Support for new claim 97, can be found at least on page 10, paragraph [0026], lines 7-9 of the specification.

Support for new claim 98 can be found at least on page 10, paragraph [0026], lines 7-9 of the specification and page 29, paragraph [0050], lines 5-7 of the specification.

Rejections Under 35 U.S.C. §102

In the Office Action, the PTO rejects claims 10-16, 19-23, and 25 as anticipated by US Patent No. 6,270,958 to Olivo (hereinafter "Olivo"). Applicants request reconsideration and withdrawal of the rejection, because Olivo does not recite all the elements of the claims.

For a reference to anticipate a claim, the reference must disclose each and every element of the claim. MPEP § 706.02; MPEP § 2131. In this case, claim 10 as amended (and claims 11-16, 19-23 and 25 by dependence from claim 10) recites a method for detecting the presence, absence, or quantity of a segmented negative strand virus. The method comprises, *inter alia*, "providing a genetically engineered vertebrate cell ...wherein the cell lacks at least one nucleocapsid protein of the virus..." In contrast, Olivo teaches a method for detecting a negative strand virus which utilizes cells comprising each of the nucleocapsid proteins of the negative strand virus (see, e.g., Olivo claim 11, and column 3 line 50-column 4 line 7 ("...the invention provides a method for detecting a negative-strand RNA virus in a sample which comprises culturing the above-described cell [comprising nucleotide sequences encoding each of the

nucleocapsid proteins] for a time sufficient to synthesize the minigenome or miniantigenome RNA *and the nucleocapsid proteins*, then incubating the cells with the sample...) (Emphasis added). Accordingly, the present claims are novel at least by virtue of utilizing cells lacking one or more proteins considered by Olivo to be necessary for a virus detection assay.

In the Office Action, the PTO further cites Nakagawa et al., J. Virology 70: 6390-6394, 1996 to support the rejection (Hereinafter "Nakagawa '96"). The PTO asserts that Nakagawa '96 teaches that "PB2 is not required for replication or transcription of uncapped poly-A RNA, and that despite the absence of influenza viral protein PB2, PB1, and PA alone can support and are sufficient for viral RNA synthesis in replication of the genome."

In response, Applicants assert that Olivo in view of Nakagawa '96 does not obviate the present claims, because Nakagawa '96 does not teach expression of a polypeptide encoded by a reporter gene in response to infection of a cell, but instead only addresses transcription. In addition, the abstract of Nakagawa '96 cites a previous publication by Nakagawa et al., J. Virology 69: 728-733, 1995 (hereinafter "Nakagawa '95"), which teaches that expression of a reporter polypeptide in a cell requires all the viral nucleocapsid proteins. Nakagawa '95 describes a first cell line ("clone 76") which expresses three RNA polymerase genes PB1, PB2 and PA as well as the NP gene of influenza virus, and second a cell line ("clone 64") which expresses PA, PB1, and NP but not PB2. Nakagawa '95 reports that adding exogenous RNA encoding chloramphenicol acetyltransferase (CAT, a commonly used reporter polypeptide) leads to detectable CAT activity in clone 76 cells but not in clone 64 cells (see, e.g., Fig. 4 on page 731). Thus, in spite of the presence of RNAs encoding a reporter polypeptide as well as three nucleocapsid proteins of the influenza virus, detectable translation product of the RNA encoding a reporter polypeptide was not observed in Nakagawa '95. Nakagawa '96 does not make any

statements that contradict anything in Nakagawa '95, and in fact states on page 6390, as cited by the examiner, that "PB2 subunit is not required for *replication or transcription to yield uncapped poly(A)+ -RNA*." (emphasis added). This passage states nothing about translation. Furthermore, except for concluding remarks on p. 6393, Nakagawa '96 is silent about expression or detection of a reporter polypeptide, and these concluding remarks state that prior reverse genetic studies on Vaccinia virus and SV-40 virus indicated that "... CAT activity was examined after the transfection of chimeric NS-CAT RNA in cells that simultaneously expressed all three polymerase subunits and NP. *The activity was not observed in cells that did not express any of these proteins*."(Emphasis added). Accordingly, Olivo does not teach each and every element of any of claims 10-16, 19-23, and 25, even in view of Nakagawa '95 and '96. If anything, Olivo and Nakagawa '95 and '96 show that the inventors of the present claims obtained unexpected results, in that these references suggest that a negative strand RNA virus would not be expected to be detectable in a cell-based assay such as assays disclosed in the present application in which the host cell does not express viral polypeptides PB1, PB2, PA and NP prior to infection. Applicants, therefore, request withdrawal of the rejection of claims 10-16, 19-23, and 25 under 35 U.S.C. § 102.

Rejections Under 35 U.S.C. §103

Claims 1-3, 6-9 and 18 stand rejected under 35 U.S.C. § 103(a) as unpatentable over Olivo, in view of a definition of "nucleocapsid" found by the PTO in an on-line dictionary. Applicants respectfully traverse the rejection because the PTO has not established a *prima facie* case for obviousness under 35 U.S.C. § 103. In addition, Applicants traverse the rejection

because a claim in dependent form is construed to incorporate by reference all the limitations of the claim to which it refers. 35 U.S.C. § 112, fourth paragraph.

In order to establish a *prima facie* case for obviousness, the PTO must show, inter alia, that there is a reasonable expectation of success, and that the cited references teach or suggest all the claim limitations. MPEP § 2142. In the present case, the PTO has shown neither a reasonable expectation of success nor a teaching or suggestion of all the claim elements of any of claims 1-3, 6-9 and 18. When properly construed as required by statute, claims 1-3, 6-9 and 18 each include as an element, cells which lack at least one nucleocapsid protein of the virus. In contrast, the methods of Olivo utilize cells which comprise "nucleotide sequences encoding each of the nucleocapsid proteins." (column 3, lines 50-55) The cells, by implication, comprise each of the nucleocapsid proteins. A virus detection assay in Olivo thus comprises incubating cells expressing each of the nucleocapsid proteins "for a time sufficient to synthesize the minigenome or miniantigenome RNA *and the nucleocapsid proteins*, then incubating the cells with the sample and detecting expression of the reporter gene product" (column 3 line 65-column 4 line 4)(Emphasis added). Applicants contend that notwithstanding the dictionary definition of "nucleocapsid" provided by the PTO, Olivo does not disclose all the elements of the claimed methods. Furthermore, Olivo provides no reasonable expectation of success for the claimed methods, in that Olivo discloses methods which *require* expression of all the nucleocapsid proteins in cells prior to contacting the cells with a test sample. Applicants, therefore, request reconsideration and withdrawal of the rejection of claims 1-3, 6-9 and 18 under 35 U.S.C. § 103.

Claim 24 stands rejected under 35 U.S.C. § 103(a) as unpatentable over Olivo in view of Neumann et al., Virology 202: 477-479, 1994 ("Neumann"). Applicants respectfully traverse the rejection, because the PTO has not established a *prima facie* case for obviousness under 35

U.S.C. § 103, and because a claim in dependent form is construed to incorporate by reference all the limitations of the claim to which it refers. 35 U.S.C. § 112, fourth paragraph.

In order to establish a *prima facie* case for obviousness, the PTO must show, inter alia, that there is a reasonable expectation of success, and that the cited references teach or suggest all the claim limitations. MPEP § 2142. In this case, the PTO has shown neither a reasonable expectation of success nor a teaching or suggestion of all the claim elements in the cited references.

When properly construed in accordance with statute, claim 24 includes all the elements of claim 10. As noted supra in reference to claim 10, Olivo does not disclose methods of detecting a segmented negative strand RNA virus which utilize cells lacking at least one nucleocapsid protein of the virus. In addition, Neumann also does not disclose an assay for a virus using cells lacking at least one nucleocapsid protein of the virus. To the contrary, Neumann states, starting from the last paragraph on page 477, that cells "were *first infected* with influenza A/Asia/57 (H2N2) or virus A/FPV Bratislava (H7N1), respectively, and *transfected 2 hr later* with plasmid pHL925 DNA [encoding a CAT reporter]." (Emphasis added.) Infection of cells with virus prior to transfection with a reporter construct indicates that the infected cells comprised viral nucleocapsid proteins by the time of transfection. Neumann, therefore, does not describe a method for detecting a virus which involves cells lacking at least one nucleocapsid protein of the virus. Accordingly, neither Olivo nor Neumann, viewed singly or in combination, teach the method of claim 24. Furthermore, neither of these references, either alone or in combination, provide a reasonable expectation of success, at least because neither reference suggests that a reporter polypeptide can be expressed in a cell in response to viral infection, from an RNA present in the cell prior to infection in the absence of at least one nucleocapsid protein of the

virus. Reconsideration and withdrawal of the rejection of claim 24 under 35 U.S.C. § 103 is, therefore, respectfully requested.

Claims 4-5 and 17 stand rejected under 35 U.S.C. § 103(a) as unpatentable over Olivo in view of Fodor et al., J. Virology 73: 9679-9682, 1999 ("Fodor"). Applicants respectfully traverse the rejection, because the PTO has not established a *prima facie* case for obviousness under 35 U.S.C. § 103, and because a claim in dependent form is construed to incorporate by reference all the limitations of the claim to which it refers. 35 U.S.C. § 112, fourth paragraph.

In order to establish a *prima facie* case for obviousness, the PTO must show, inter alia, that there is a reasonable expectation of success, and that the cited references teach or suggest all the claim limitations. MPEP § 2142. In this case, the PTO has shown neither a reasonable expectation of success nor a teaching or suggestion of all the claim elements in the cited references.

When properly construed in accordance with statute, claims 4-5 and 17 include all the elements of claims 1 and 10, respectively. Hence, each of these claims include methods which utilize, *inter alia*, cells lacking at least one nucleocapsid protein of a virus. As noted supra, Olivo does not disclose methods of detecting a segmented negative strand RNA virus which utilize cells lacking at least one nucleocapsid protein of the virus. In addition, Fodor does not disclose methods of detecting a segmented negative strand RNA virus which utilize cells lacking at least one nucleocapsid protein of the virus. Fodor, instead, discloses rescue of Influenza A virus from recombinant DNA by expressing in a cell, inter alia, influenza viral polypeptides PA, PB1 and PB2 and NP. Hence, neither Olivo nor Fodor, either alone or in combination, teach all the elements of any of claims 4, 5 and 17. Furthermore, neither of these references, either alone or in combination, provide a reasonable expectation of success, at least because neither reference

suggests that a reporter polypeptide can be expressed in a cell in response to viral infection, from an RNA present in the cell prior to infection. Reconsideration and withdrawal of the rejection of claims 4-5 and 17 under 35 U.S.C. § 103 is, therefore, respectfully requested.

Conclusion

For the foregoing reasons, Applicants respectfully request reconsideration and withdrawal of rejections of the claims. It is believed that the claims as currently presented are in a condition for allowance and such favorable action is respectfully requested. If any questions arise or if any issues remain to be resolved, it is requested that the Examiner contact the undersigned attorney.

Respectfully submitted,

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The viral polymerase mediates adaptation of an avian influenza virus to a mammalian host

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Mammalian influenza viruses are descendants of avian strains that crossed the species barrier and underwent further adaptation. Since 1997 in southeast Asia, H5N1 highly pathogenic avian influenza viruses have been causing severe, even fatal disease in humans. Although no lineages of this subtype have been established until now, such repeated events may initiate a new pandemic. As a model of species transmission, we used the highly pathogenic avian influenza virus *SC35* (H7N7), which is low-pathogenic for mice, and its lethal mouse-adapted descendant *SC35M*. Specific mutations in *SC35M* polymerase considerably increase its activity in mammalian cells, correlating with high virulence in mice. Some of these mutations are prevalent in chicken and mammalian isolates, especially in the highly pathogenic H5N1 viruses from southeast Asia. These activity-enhancing mutations of the viral polymerase complex demonstrate convergent evolution in nature and, therefore, may be a prerequisite for adaptation to a new host paving the way for new pandemic viruses.

evolution | pathogenicity

Influenza A viruses have the potential to cause devastating pandemics (1) and may cross species barriers as a whole and adapt to a new host (2, 3). Direct transmissions of highly pathogenic avian influenza A viruses (HPAIV) of subtype H5N1 to humans were first documented 1997 in Hong Kong (4, 5). Since 2003, H5N1 strains again have occasionally caused fatal disease in humans (6). Although these viruses have not established a lineage in humans until now, such repeated events might initiate a new pandemic.

In this study, we investigated the adaptation of an HPAIV to a mammalian host. As model served the virus *SC35* and its mouse-adapted descendant *SC35M*. *SC35* was derived from A/Seal/Massachussetts/1/80 (H7N7) by serial passages in chicken embryo cells, thereby acquiring a multibasic cleavage site in its hemagglutinin (HA) (7) and becoming 100% lethal for chickens. *SC35* was then passaged 11 times in mouse lung (H. Scheiblauer, personal communication), yielding the mouse-adapted variant *SC35M* (8). In contrast to *SC35*, which is low-pathogenic for mice, *SC35M* is highly pathogenic for both mice and chickens. *SC35M* and *SC35* therefore provide a suitable system to elucidate the molecular basis of host change and enhanced virulence in mammals.

SC35 and *SC35M* differ mainly by mutations in the polymerase proteins (PB2, PB1, and PA) and in the nucleoprotein (NP). *SC35M* has a considerably higher polymerase activity in mammalian cells than *SC35*. This increased activity correlates with increased virulence in mice. Similar polymerase mutations were found in unrelated strains, especially H5N1 HPAIV and human isolates.

Materials and Methods

Cells and Viruses. We grew 293T human embryonic kidney cells in DMEM supplemented with 10% FCS and Madin–Darby canine kidney (MDCK) cells in MEM containing 10% FCS. We produced chicken embryo fibroblasts by digesting pieces of 11-day-old chicken embryos in buffered saline containing 2.5% trypsin (GIBCO/BRL); the cells were then cultured in DMEM with 10% FCS. We propagated influenza A viruses in 11-day-old embryonated chicken eggs. The growth curves were determined in three independent experiments by plaque titration (9). SC35, SC35M, and their mutants were handled under biosafety level 3 conditions.

Plasmids. To clone all eight genes of *SC35* and *SC35M*, we amplified each segment by RT-PCR from isolated RNA (10) and ligated it into plasmid pHW2000 (11). For reporter assays, we used an influenza minigene containing luciferase as reporter protein (pPolI-NP-luc) and for standardization a β-gal-expressing plasmid (pRSV-β-gal). The plasmid pPolI-NP-Luc expresses a negative-sense RNA transcript carrying the complete ORF of the firefly luciferase gene (accession no. AF053462) flanked by the noncoding regions of the NP segment of influenza A/WS/33 virus (23 nt of the viral RNA 5′ end and 45 nt of the 3′ end; accession no. M30746).

Mutagenesis. We introduced mutations of *SC35M* into the cDNAs from *SC35* or vice versa by site-directed mutagenesis with the QuikChange mutagenesis kit (Stratagene) according to the manufacturer's protocol. We verified the presence of the introduced mutation and the absence of additional unwanted mutations by sequencing of the whole cDNA.

Rescue of Recombinant Viruses. We transfected 293T cells with a mixture of eight plasmids encoding all eight influenza gene segments (1 μg each) and Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer. We removed the medium 6 h later and replaced it with DMEM containing 0.2% BSA. After another 42 h, we plated the supernatant onto MDCK cells. We plaque-purified rescued viruses twice and inoculated them in 11-day-old hen eggs for propagation of stock virus. We ascertained the identity of propagated mutant viruses by sequencing amplicons of each viral gene segment by using RT-PCR.

Growth Curves. We inoculated chicken embryo fibroblasts, Vero cells, A549 (human lung epithelial cell line) and LA-4 (mouse adenoma cells), respectively, with virus at a multiplicity of infection of 10^{-4} and incubated them in the appropriate medium containing 0.2% BSA at 37°C. At time points 0, 8, 24, 48, 72, 96, and 120 h, we collected supernatants and determined the plaque titer on MDCK cells.

Luciferase Assay After Transfection. We transfected 293T cells with the pHW2000 plasmids constructs encoding the polymerase genes PB2, PB1, PA, and NP and the pPol1-NP-Luc and pRSV-β-gal plasmids (1 μg each) plus Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer. After 6 h, we replaced the medium with DMEM (10% FCS). At 24 h after transfection, we

Conflict of interest statement: No conflicts declared.

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Abbreviations: HA, hemagglutinin; HPAIV, highly pathogenic avian influenza virus; MDCK, Madin–Darby canine kidney; NA, neuraminidase; NP, nucleoprotein; SGR, single-gene reassortant.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. DQ266094–DQ266101).

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Table 1. Recombinant viruses

	Genotype									
	PB1		PB2			PA	HA	NP	NA	
Virus	13	678	333	701	714	615	340	319	328	LD_{50}
SC35	L	S	T	D	S	K	G	N	A	>6.0
SC35M	P	N	I	N	R	N	R	K	S	2.8
SC35-PB1$_{SC35M}$	P	N	T	D	S	K	G	N	A	5.6
SC35-PB2$_{SC35M}$	L	S	I	N	R	K	G	N	A	>6.0
SC35-PA$_{SC35M}$	L	S	T	D	S	N	G	N	A	6.0
SC35-HA$_{SC35M}$	L	S	T	D	S	K	R	N	A	>6.0
SC35-NP$_{SC35M}$	L	S	T	D	S	K	G	K	A	>6.0
SC35-NA$_{SC35M}$	L	S	T	D	S	K	G	N	S	>6.0
SC35-PB1$_{13P}$	P	S	T	D	S	K	G	N	A	>6.0
SC35-PB1$_{678N}$	L	N	T	D	S	K	G	N	A	>6.0
SC35-PB2$_{701N}$	L	S	T	N	S	K	G	N	A	6.0
SC35-PB2$_{714R}$	L	S	T	D	R	K	G	N	A	6.0
SC35-PB2$_{701N+714R}$	L	S	T	N	R	K	G	N	A	3.5
SC35-PB2$_{701N}$-NP$_{SC35M}$	L	S	T	N	S	K	G	K	A	5.5
SC35-PB2$_{714R}$-NP$_{SC35M}$	L	S	T	D	R	K	G	K	A	5.5
SC35-PB2$_{701N+714R}$-NP$_{SC35M}$	L	S	T	N	R	K	G	K	A	6.0
SC35M-PB1$_{13L}$	L	N	I	N	R	N	R	K	S	4.2
SC35M-PB1$_{678S}$	P	S	I	N	R	N	R	K	S	3.5
SC35M-PB2$_{333T}$	P	N	T	N	R	N	R	K	S	1.4
SC35M-PB2$_{701D}$	P	N	I	D	R	N	R	K	S	3.3
SC35M-PB2$_{714S}$	P	N	I	N	S	N	R	K	S	>6.0
SC35M-HA$_{monobasic}$	P	N	I	N	R	N	MB	K	S	4.6

SC35, SC35M, SGRs, and single-point mutants with their amino acid exchanges and mouse LD_{50}. Amino acid replacements in *SC35M* are shown in bold. MB, monobasic.

harvested and lysed the cells. We then assayed luciferase activity by using the Luciferase assay system detection kit (Promega). This assay was standardized against the β-gal activity (resulting from cotransfection of the pRSV-β-gal plasmid), which was determined with a β-gal enzyme assay system (Promega). The baseline value is the result of the cotransfection of pPol1-NP-Luc plus pRSV-β-gal and was subtracted from each measurement. Each luciferase activity value is the average of four independent experiments.

Luciferase Assay with Recombinant Virus. We transfected 293T cells with 1 μg of pPol1-NP-Luc plasmid and for standardization with 1 μg of pRSV-β-gal plasmid plus Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer. For infection, we washed the cells with PBS and inoculated them with influenza virus at a multiplicity of infection of 0.1. After 45 min, we washed the cells twice with PBS 10 h after transfection and incubated them with DMEM (0.2% BSA) for 14 h. We then harvested the cells 14 h after infection and assayed luciferase activity.

Animal Experiments. The animal experiments were performed according to the guidelines of the German Animal Protection law. All animal protocols were approved by the relevant German authorities, the Regierungspräsidium Tübingen and the Regierungspräsidium Giessen. We anesthetized 4- to 6-week-old, female BALB/C mice (obtained from the animal breeding facility of the Friedrich-Loeffler-Institut and bought from Charles River Laboratories) with 100 mg/kg ketamine/10 mg/kg xylazine and inoculated them intranasally with 50 μl of infectious virus diluted in PBS. We determined the LD_{50} from groups of five mice inoculated with serial 10-fold dilutions of virus (10^6 plaque-forming units as the highest dosage) and calculated the LD_{50} as described in ref. 12.

Tree Calculation. We downloaded the nucleotide sequences from the Los Alamos Influenza Database (virus names and accession numbers in Table 3, which is published as supporting information on the PNAS web site) (13) and aligned them by using the PILEUP program of the WISCONSIN package, version 10.2 (Genetics Computer Group, Madison, WI). For tree calculation, we performed a neighbor-joining analysis following bootstrap calculation (1,000 samples) by using the programs SEQBOOT, DNADIST, NEIGHBOR, and CONSENSE from PHYLIP 3.6 (14). The regions of PB2, PA, and NP sequences used for calculation were nucleotides 1039–2268, 51–2169, and 46–1542, respectively.

Results

Generation of Recombinant *SC35* and *SC35M* Viruses. Recombinant *SC35* and *SC35M* viruses were generated by reverse genetics (11) after their genes were cloned into the plasmid pHW2000 (11). By using appropriate plasmid combinations, we then obtained single-gene reassortants (SGR) containing seven genes of *SC35* plus one of *SC35M* and vice versa. According to the nomenclature we used, an SGR of *SC35* with the NP gene of *SC35M* was designated SC35-NP$_{SC35M}$. After directed mutagenesis, we also rescued recombinant viruses with point mutations. A virus containing the *SC35* genes with the mutation L13→P, observed in PB1 of *SC35M*, was named SC35-PB1$_{13P}$. Its reciprocal counterpart is the virus *SC35M*-PB1$_{13L}$. The low-pathogenic *SC35* differs from the highly pathogenic *SC35M* by 9 amino acid exchanges. There are three amino acid exchanges in PB2, two in PB1, and one in PA, NP, HA, and neuraminidase (NA), respectively (Table 1).

Growth in Chicken and Mammalian Cells. We studied the growth of *SC35* and *SC35M* in avian and mammalian cells. In chicken embryo fibroblasts, both viruses grow to high titers (≈10^7 plaque-forming units/ml) without notable difference (Fig. 1*A*). This result corresponds to high pathogenicity of *SC35* and *SC35M* in chickens (7, 8). In mammalian cell lines, like monkey kidney cells (Vero) (Fig. 1*B*), mouse lung adenoma cells (LA-4) (Fig. 1*C*), and human epithelial lung cells (A549) (Fig. 1*D*), the mouse-adapted *SC35M* grows to titers 2–4 logs higher than *SC35*. This difference demonstrates a considerable growth advantage of *SC35M* in mammalian cells.

Activity of Reconstituted Ribonucleoprotein Complexes. To investigate whether the *SC35M* polymerase mutations change enzymatic activity, we analyzed reconstituted ribonucleoprotein complexes in 293T cells by a luciferase minigene assay. *SC35M* has a polymerase

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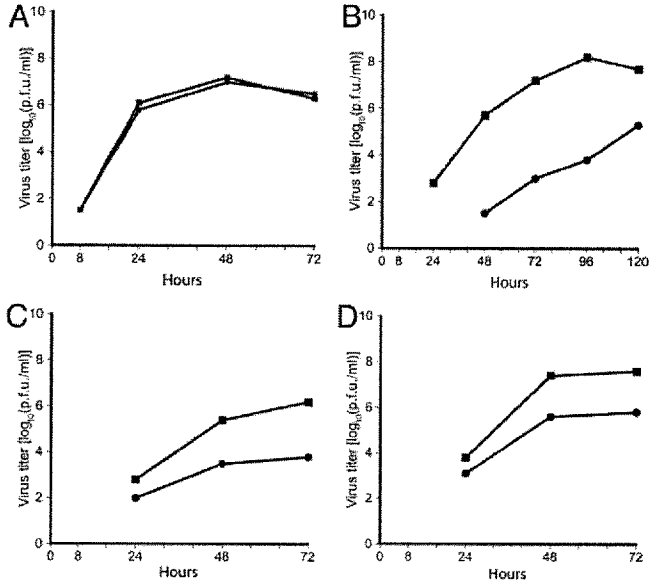

Fig. 1. Growth in avian and mammalian cells. Growth curves of *SC35* (●) and *SC35M* (■) in chicken embryo fibroblasts (CEF) (*A*), monkey kidney cells (Vero) (*B*), mouse lung adenoma cells (LA-4) (*C*), and human epithelial lung cells (A549) (*D*). Cells were inoculated at a multiplicity of infection of 10^{-4}. The plaque titers were determined at appropriate time points on MDCK cells. Each curve is the average of two independent experiments.

activity of 600% compared with *SC35* (Fig. 2*A*, columns a and b). To assign this increase to single mutations, we investigated several combinations. The *SC35M* PB2 and NP increased polymerase activity to 300% and 220%, respectively, whereas PB1 and PA did not (Fig. 2*A*, columns c–f). PB2 and NP of *SC35M* together with PA and PB1 of *SC35* have a similar polymerase activity of 600% as the authentic polymerase complex of *SC35M* (Fig. 2*A*, column g). By contrast, the reciprocal constellation of *SC35* PB2 and NP with *SC35M* PB1 and PA showed the low polymerase activity of *SC35* (Fig. 2*A*, column h), stressing the importance of the combination of PB2 and NP from *SC35M* for enhanced replication. Replacing one gene of the *SC35M* polymerase complex lowered its activity (Fig. 2*A*, column i–l). This finding implies that, in addition to PB2 and NP, the homologous combination of PB1 and PA of *SC35M* (Fig. 2*A*, column b) or *SC35* (Fig. 2*A*, column g) is required. Thus, PB1 and PA have to match for the high polymerase activity of *SC35M*. We then introduced the three *SC35M* PB2 mutations into the *SC35* PB2 plasmid. PB2 701N and 714R each increase activity up to 300% (Fig. 2*B*, columns d and e); stepwise combination of 701N and 714R in PB2 and 319K in NP leads to further rises (Fig. 2*B*, columns g, h, i, and l). However, PB2 333I suppresses polymerase activity in any combination with 701N and 714R in PB2, or NP 319K (Fig. 2*B*, columns c, f, j, k, m, and n). Taken together, mutations in *SC35M* PB2 (701N and 714R) and NP (319K) considerably enhance polymerase activity in mammalian cells in an additive manner. In contrast, PB2 333I is a down-regulating mutation.

Lys-627 in PB2 is a well known determinant of mammalian host range (15). The HPAIV *SC35* has glutamic acid in this position like most avian isolates. The amino acid replacement 627K in the PB2 plasmid of *SC35* raised the polymerase activity to 2,000% (Fig. 4, which is published as supporting information on the PNAS web site). Thus, this host range mutation, which is not observed in *SC35M*, also enhances polymerase activity.

Polymerase Activity of Recombinant Virus. In the luciferase-minigene assay performed with live virus, *SC35M* showed an increase in

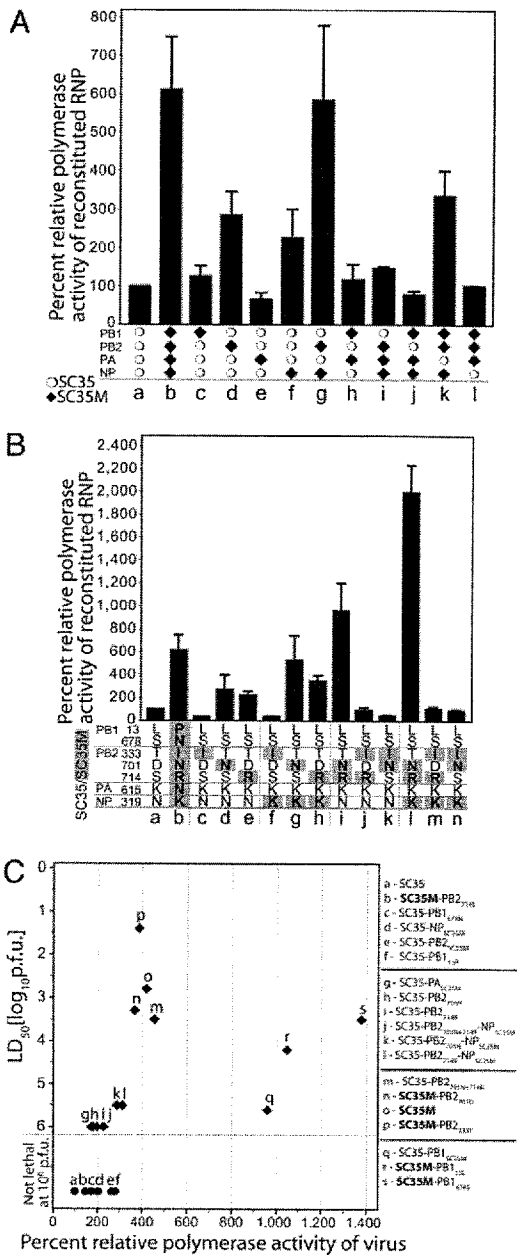

Fig. 2. Enhanced polymerase activity of *SC35M*. (*A* and *B*) Polymerase activity of ribonucleoprotein complexes reconstituted from PB1, PB2, PA, and NP plasmids of *SC35*, *SC35M*, and their combinations (*A*) and of their single-point mutants (*B*). (*C*) Correlation of polymerase activity of recombinant virus with virulence. The mouse LD_{50} of viruses was plotted (inverted axis) against their polymerase activities. The polymerase activities were determined by luciferase reporter assay in four independent experiments.

polymerase activity of 420% compared with *SC35* (Fig. 2*C*; see also Fig. 5, columns a and b, which is published as supporting information on the PNAS web site). SGRs containing one polymerase gene segment of *SC35M*, SC35-$PB1_{SC35M}$, SC35-$PB2_{SC35M}$, SC35-PA_{SC35M}, and SC35-NP_{SC35M} have increased polymerase activity ranging between 200% and almost 1,000% (Figs. 2*C* and 5, columns c, f, g, and h). The PB1 single-point mutants SC35-$PB1_{13P}$ and SC35-$PB1_{678N}$ showed enhanced activities of 270% and 170%, respectively (Figs. 2*C* and 5, columns d and e). However, the reciprocal *SC35M* PB1 mutants, SC35M-$PB1_{13L}$ and SC35M-

$PB1_{678S}$, possess activities of even 1,000% and 1,400% (Figs. 2*C* and Fig. 5, columns o and p). The PB2 point mutants SC35-$PB2_{701N}$ and SC35-$PB2_{714R}$ have an increase in activity (180% and 170%), even higher in combination with *SC35M* NP: SC35-$PB2_{701N}$-NP_{SC35M} and SC35-$PB2_{714R}$-NP_{SC35M} (310% and 290%) (Figs. 2*C* and 5, columns i–l). Combination of the two PB2 mutations in SC35-$PB2_{701N+714R}$ further enhances polymerase activity to 450% but not with *SC35M* NP as in SC35-$PB2_{701N+714R}$-NP_{SC35M} (230%) (Figs. 2*C* and 5, columns m and n). Therefore, efficient polymerase activity is achieved by the combination of PB2 701N or PB2 714R with *SC35M* NP. Removal of PB2 701N in *SC35M* (SC35M-$PB2_{701D}$) did not change polymerase activity. However, replacement of 714R as in SC35M-$PB2_{714S}$ lowered it (150%) (Figs. 2*C* and 5, columns r and s). Therefore, PB2 714R is essential for high polymerase efficiency. SC35M-$PB2_{333T}$ without the suppressing mutation 333I (Fig. 2*B*, columns c, f, j, k, m, and n) reaches the same maximum activity (400%) as *SC35M* (Figs. 2c and 5, columns b and q) but does so 3 h earlier (Fig. 6, which is published as supporting information on the PNAS web site). Only this virus presented a striking cytopathic effect in 293T cells at time of luciferase assay. Thus, the increased polymerase activity of SC35M-$PB2_{333T}$ results in accelerated virus replication and enhanced cytotoxicity. PB2 333T, although further increasing the polymerase activity, may be detrimental to the virus fitness *in vivo*. Such a disadvantage may be the reason why threonine was not observed at position PB2 333 after mouse adaptation.

Taken together, PB2 701N and 714R, PA 615N, and NP 319K enhance polymerase activity of the virus recombinants moderately; PB1 13P and 678N cause a dramatic increase.

Virulence in Mice. To elucidate the contribution of *SC35M* mutations to virulence in mice, we determined the LD_{50}. *SC35M* has an LD_{50} of 2.8 in contrast with *SC35*, which, at 10^6 plaque-forming units, is nonlethal but causes weight loss for 2 d (Table 1). The SGRs carrying one gene of *SC35M* have an LD_{50} of >6.0 except SC35-PA_{SC35M} (LD_{50} = 6.0) and SC35-$PB1_{SC35M}$ (LD_{50} = 5.6). This considerably reduced virulence of SGRs compared with *SC35M* indicates that these mutations are required in combination for a highly pathogenic phenotype; if present alone, they have no enhancing effect. By contrast, only if these mutations are lost, as in their reciprocal counterparts, do they alter the phenotype. Thus, SC35-$PB1_{13P}$ and SC35-$PB1_{678N}$ are not pathogenic. However, their reciprocal counterparts, SC35M-$PB1_{13L}$ and SC35M-$PB1_{678S}$, are more virulent (LD_{50} = 4.2 and 3.5). Remarkably, the PB2 mutations seem to be additive. The single-point mutants SC35-$PB2_{701N}$ and SC35-$PB2_{714R}$ have an LD_{50} of 6.0; combination of these mutations in SC35-$PB2_{701N+714R}$ raised virulence (LD_{50} = 3.5). Their reciprocal counterparts, SC35M-$PB2_{701D}$ and SC35M-$PB2_{714S}$, have lower virulence than *SC35M*. Remarkably, SC35M-$PB2_{714S}$ did not present a detectable LD_{50}, highlighting the importance of PB2 714R in *SC35M* for high virulence. The single-point mutant SC35M-$PB2_{333T}$ has an LD_{50} of 1.4, which is even lower than that of *SC35M* (2.8). A reciprocal virus SC35-$PB2_{333I}$ harboring the down-regulating mutation could never be rescued, despite several attempts, which indicates that such a virus is not viable because of reduced polymerase activity, supporting the concept that PB2 333I is a down-regulating mutation. Combination of PB2 mutations 701N and 714R each with the *SC35M* NP gene (SC35-$PB2_{701N}$-NP_{SC35M}, SC35-$PB2_{714R}$-NP_{SC35M}) increased virulence compared with SC35-$PB2_{701N}$ and SC35-$PB2_{714R}$, except SC35-$PB2_{701N+714R}$-NP_{SC35M} (Table 1).

Especially, the mutations in PB2 and NP have an additive effect on pathogenicity, as in SC35-$PB2_{701N}$-NP_{SC35M}, SC35-$PB2_{714R}$-NP_{SC35M}, and SC35-$PB2_{701N+714R}$. In contrast, PB2 714R alone introduced into *SC35* does not raise pathogenicity. Nevertheless, PB2 714R is essential for *SC35M* pathogenicity because substitution with 714S renders the virus with the remaining mutations (SC35M-$PB2_{714S}$) low-pathogenic. This dramatically altered phenotype indicates that PB2 714R is indispensable for adaptation in the context of the other mutations.

Table 2. Database search for *SC35M* mutations in natural strains

Viral gene	Amino acid position	Total no. sequences	No. same as *SC35*	No. same as *SC35M*	No. different
PB2	701	694	664 (Asp)	30 (Asn)	—
	714	694	674 (Ser)	—	16 (Ile), 4 (Gly)
PA	615	650	600 (Lys)	1 (Asn)	49 (Arg)
NP	319	1,101	1,082 (Asn)	19 (Lys)	—

Comparison of amino acids in sequences of natural strains at positions corresponding to *SC35M* mutations. Shown are the number of all sequences in the database, the number of sequences with the same amino acid as *SC35* and *SC35M*, and the number of sequences with a different amino acid at the same position (with the amino acids shown in parentheses). —, not applicable.

The SGRs containing the surface glycoproteins of *SC35M*, SC35-HA_{SC35M}, and SC35-NA_{SC35M} had no increased virulence (Table 1). The substitution in the NA did not change NA activity measured in different assays *in vitro* (data not shown). The HA cleavage mutant SC35M-$HA_{monobasic}$ is less virulent (LD_{50} = 4.6) than *SC35M* (LD_{50} = 2.8). Therefore, both the polybasic cleavage site and the polymerase mutations contribute to high pathogenicity of *SC35M*.

Correlation of Polymerase Activity with Pathogenicity. Pathogenicity increases with rising polymerase activity (Fig. 2*C*). The most virulent viruses (SC35-$PB2_{701N+714R}$, SC35M-$PB2_{701D}$, SC35M, and SC35M-$PB2_{333T}$) have activities ≈4-fold higher than avirulent viruses. However, three viruses (SC35-$PB1_{SC35M}$, SC35M-$PB1_{13L}$, and SC35M-$PB1_{678S}$) with excessive activity (900%) are not of the highest virulence in mice. Thus, it appears that high pathogenicity requires a polymerase activity optimum.

***SC35M* Polymerase Mutations in Mammalian Isolates.** To find out whether the amino acid exchanges observed in *SC35M* occur also in other strains, we looked at corresponding positions in published sequences (13). These positions are highly conserved and in most instances contain the same amino acid as *SC35*. However, some isolates have mutations at positions PB2 701 and 714, PA 615, or NP 319 as does *SC35M*, although the resulting amino acid exchanges were not always identical (Table 2). Interestingly, most of these strains are mammalian viruses that have been recently transmitted from birds and are still in adaptation to the new host. From their sequences and those of representative strains (16–18), phylogenetic trees were calculated to see whether the *SC35M*-like mutations are inherited in related lineages or whether they occur independently.

Among the 694 sequences analyzed, 664 contained aspartate as *SC35* and 30 asparagine at position PB2 701 (Table 2). PB2 701N is present in 28 mammalian and two related avian isolates (Fig. 3*A*) and comprise several clades of H1N1, H1N2, and H3N2 swine isolates, H3N8 and H7N7 equine isolates, the mouse-adapted variant of pandemic *A/HK/1/68* (H3N2), and the clade of H5N1 HPAIV from southeast Asia, including human isolates, like *A/HK/97/98* and *A/HK/488/97*. Moreover, PB2 701N was also found in *A/VietNam/3046/2004* (6) and *A/Tiger/Suphan./Thail./Ti-1/04* (19), which were isolated during outbreaks in Vietnam and Thailand. Remarkably, among the strains found is *A/Swine/Germany/2/81*, an avian-like swine virus (2). This isolate belongs to a lineage originating from an avian virus that was transmitted as a whole into swine (20). Thus, PB2 701N might support the adaptation to the mammalian host.

At position PB2 714, most viruses have the serine present in *SC35*, whereas amino acid exchanges are observed in four mammalian strains and 16 avian H5N1 isolates from 2001 (Table 2). The latter ones are highly pathogenic for chickens and quails, and they

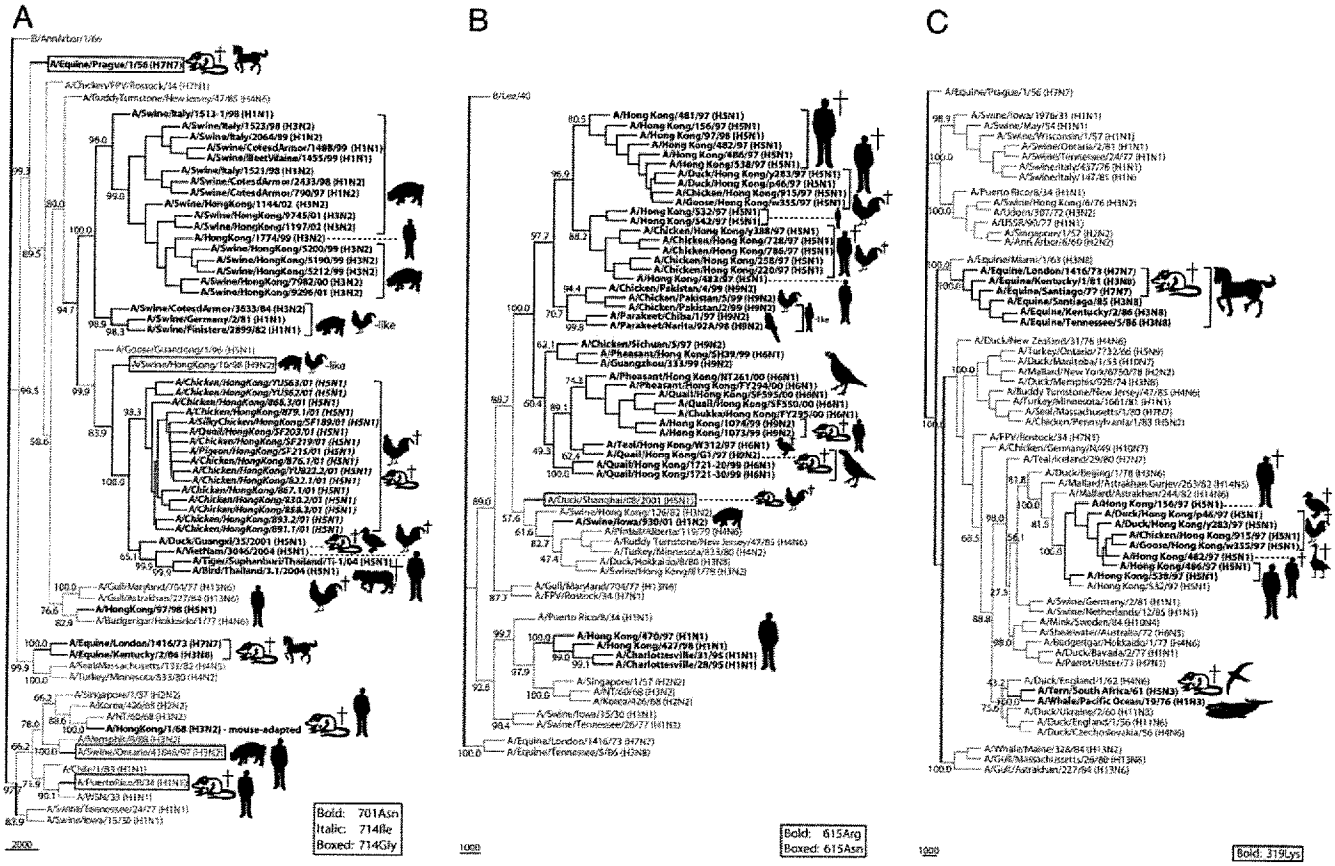

Fig. 3. Influenza strains convergent with *SC35M* polymerase mutations. Shown are phylogenetic trees from PB2 (*A*), PA (*B*), and NP (*C*) genes of strains convergent with *SC35M* polymerase mutations (black) and representative strains without polymerase mutations (gray). Consensus trees are from neighborhood-joining analyses from bootstrap samples. Numbers at nodes are bootstrap values.

replicate well in mouse lung in contrast to their precursors, the Gs/Gd-like strains (21). One of these strains, *A/Goose/Guangdong/1/96* (22), has serine at position PB2 714 as does *SC35* (Fig. 3*A*). In contrast to the Gs/Gd-like viruses, the H5N1 isolates appear to have increased pathogenicity for mammals (21), supporting the view that PB2 714I is a host range marker.

PA 615N was found only in *A/Duck/Shanghai/08/2001* (H5N1). Among 650 isolates, 600 isolates harbor lysine as *SC35*, 1 isolate harbors Asparagine, and 49 isolates harbor arginine (Table 2). PA 615R was found in several clusters of human H1N1, H5N1, and H9N2 isolates (Fig. 3*B*), including H5N1 HPAIV isolates of known high pathogenicity for mice and humans, like *A/HK/483/97*, *A/HK/485/97*, and *A/HK/491/97* (23). The strains *A/Teal/HK/W312/97* (H6N1) and *A/Quail/HK/G1/97* (H9N2), proposed donors of internal genes of H5N1 viruses (24–26), also were found, emphasizing the relevance of PA 615R for host change.

NP 319K in *SC35M*, was observed in 19 of 1,101 sequences (Table 2 and Fig. 3*C*); the others contain asparagine. Fourteen isolates are human and equine strains. Five isolates are highly pathogenic for chickens and closely related to the highly pathogenic human H5N1 isolate *A/HK/156/97* (27). Remarkably, this virus and other human H5N1 isolates (*A/HK/482/97*, *A/HK/486/97*, and *A/HK/538/97*) (23, 28) harboring NP 319K also carry PA 615R. Therefore, these changes in combination might be responsible for higher virulence in mammals. In general, the phylogenetic trees of PB2, PA, and NP demonstrate (Fig. 3) that the amino acids corresponding to *SC35M* exchanges are distributed over unrelated clusters. Therefore, these changes occurred independently. This finding demonstrates convergent evolution of the polymerase complex in different virus lineages during adaptation to the mammalian host.

Discussion

The molecular basis of adaptation of influenza A viruses to a new host species is poorly understood. To address this problem, we have analyzed the HPAIV *SC35* and its mouse-adapted variant *SC35M*, which differ by seven amino acid exchanges in the polymerase, one amino acid exchange in HA, and one amino acid exchange in NA. Comparison of LD_{50} in mice revealed that all polymerase mutations contribute independently to virulence. Two *SC35M* mutations in PB2 (701N and 714R) and one in NP (319K) enhance polymerase activity. The amino acids found at positions PB2 701 and 714, NP 319, and PA 615 of *SC35* are highly conserved and therefore present in most viruses analyzed. However, *SC35M*-like amino acid exchanges were found in some mammalian strains that have only recently been derived from avian viruses and in H5N1 HPAIV isolates that are also pathogenic for mammals. These observations support the concept that mutation of the polymerase complex is critical for adaptation to the new environment once the virus has been transmitted to a new host. The functional role of these mutations is not fully understood, but it is reasonable to assume that they are necessary for optimized interaction of the polymerase with the host leading to enhanced replication and transcription. Our data therefore underline that host factors are important for polymerase action. PB1 13P and 678N are localized within the PA-binding site (residues 1–25; α-domain) and the PB2-binding site (residues 600–757), respectively (29). Therefore, PB1 13P and 678N could improve the interplay of polymerase subunits and NP in a new host

environment. *SC35M* PB2 333I is located within the cap-binding region (30) and might therefore affect viral transcription. The virus without this mutation, SC35M-PB2$_{333T}$, exceeds even the high virulence of *SC35M*.

HPAIV possess polybasic cleavage sites in HA recognized by ubiquitous proteases of the subtilisin family (31–33). Such a cleavage site that is a marker for high pathogenicity is present in *SC35* and *SC35M*. However, it is interesting that SC35M-HA$_{monobasic}$ is more virulent for mice than *SC35* but less virulent than *SC35M*. This finding highlights the importance of the polybasic cleavage site and the *SC35M* polymerase mutations in *SC35M* pathogenicity.

PB2 amino acid 627 is a known determinant of replication efficiency (34, 35) and host specificity (15). The substitution PB2 E627K was attributed to increased pathogenicity in avian H5N1 influenza viruses isolated in 1997 in Hong Kong (36). Moreover, the human isolate from the chicken H7N7 virus epidemic in The Netherlands in 2003, which caused fatal disease (37), carries 627K like most human strains (15). Thus, there is evidence in several cases that PB2 627K is involved in adaptation to mammalian hosts. Introduction of 627K into the *SC35* PB2 plasmid also raised the polymerase activity excessively (2,000%), although this mutation was not found in *SC35M*. Likewise, three of the 23 recombinant viruses studied here exhibiting excessive polymerase activity (SC35-PB1, SC35M-PB1$_{13L}$, and SC35M-PB1$_{678S}$) did not show high virulence in mice. Thus, an excess of polymerase activity appears not to be compatible with high virulence, indicating that an optimum of polymerase activity is needed in the mammalian host. However, the luciferase activity as indirect marker corresponds to overall mRNA synthesis. Therefore, these mutations may lead to an imbalanced high transcription/replication ratio.

The finding of *SC35M*-like mutations in strains transmitted recently from birds and still undergoing adaptation points to convergent evolution (38) of the influenza polymerase complex in nature. Remarkably, the strains *A/Teal/HK/W312/97* (H6N1) and *A/Quail/HK/G1/97* (H9N2), proposed donors of internal genes of these H5N1 viruses (24–26), also harbor PA 615R. Human H5N1 isolates, which caused disease (23, 28), carry PA 615R and NP 319K. This combination might increase the virulence additionally. Experimental studies similar to the present work are needed to prove this concept.

Taken together, we demonstrated that the polymerase-enhancing mutations of *SC35M* contribute to increased virulence in mice and found convergent evolution of *SC35M*-like mutations in mammalian and H5N1 HPAIV strains. Therefore, the enhanced activity of viral polymerase enables HPAIV to adapt to a mammalian host. The viral polymerase may be the driving component of early evolution of influenza A viruses in a new host and pave the way for new pandemic viruses.

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The polymerase complex genes contribute to the high virulence of the human H5N1 influenza virus isolate A/Vietnam/1203/04

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H5N1 influenza viruses transmitted from poultry to humans in Asia cause high mortality and pose a pandemic threat. Viral genes important for cell tropism and replication efficiency must be identified to elucidate and target virulence factors. We applied reverse genetics to generate H5N1 reassortants combining genes of lethal A/Vietnam/1203/04 (VN1203), a fatal human case isolate, and nonlethal A/chicken/Vietnam/C58/04 (CH58) and tested their pathogenicity in ferrets and mice. The viruses' hemagglutinins have six amino acids differences, identical cleavage sites, and avian-like α-(2,3)-linked receptor specificity. Surprisingly, exchanging hemagglutinin and neuraminidase genes did not alter pathogenicity, but substituting CH58 polymerase genes completely attenuated VN1203 virulence and reduced viral polymerase activity. CH58's NS gene partially attenuated VN1203 in ferrets but not in mice. Our findings suggest that for high virulence in mammalian species an avian H5N1 virus with a cleavable hemagglutinin requires adaptive changes in polymerase genes to overcome the species barrier. Thus, novel antivirals targeting polymerase proteins should be developed.

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Abbreviations used: a.i., after inoculation; EID, egg infectious dose; HA, hemagglutinin; MDCK, Madin-Darby canine kidney; NA, neuraminidase; RG, reverse genetics; SA, α-(2,3)-linked sialic acid.

In poultry, highly pathogenic avian influenza viruses of the H5 subtype spread systemically, causing death within a few days (1). In 2004, H5N1 avian influenza was reported in mammalian species during severe outbreaks in Asia (2–6). Fatal human infections were identified in Vietnam, Thailand, Cambodia, and Indonesia. These events increased concern about a potential influenza pandemic.

Human disease caused by H5N1 viruses has been characterized by viral pneumonia with acute respiratory distress syndrome, diarrhea, liver dysfunction, and neurological symptoms (7–11). Factors responsible for this high virulence in humans are not well characterized. In chickens, hemagglutinin (HA) with multibasic amino acids is important in virus dissemination and systemic spread (12). Human A/Vietnam/1203/04 (VN1203) and avian A/Chicken/Vietnam/C58/04 (CH58) H5N1 isolates, which are both of the Z genotype, have the multibasic amino acid motif PQRERRRKKR↓G in HA and are lethal to chickens (13). However, it is not known whether other adaptive changes in HA, such as those that determine receptor specificity, are crucial for interspecies transmission and virulence. Typical avian strains are characterized by HAs that bind α-(2,3)-linked sialic acid (SA) receptors, while those of human strains bind α-(2,6)-linked SA (14). Less well understood is the role of internal gene segments. Polymerase subunit PB2 and nonstructural NS1 protein were reported to be important for high virulence of 1997 H5N1 viruses in mouse and pig models (15, 16). Virulence factors of newly emerging H5N1 Z genotype viruses have not been mapped. Mapping of viral virulence factors in different mammalian hosts would provide more information about their contribution to pathogenicity.

Ferrets are considered the best small animal model of human influenza. Mice are often used in influenza studies, but, human influenza viruses typically do not replicate in mice without adaptation, whereas they cause an acute, respiratory illness in both ferrets and humans. We and

others previously demonstrated that highly pathogenic H5N1 viruses that were isolated from birds and humans in 2004 form groups according to their pathogenicity in ferrets (13, 17). VN1203, which was isolated from a fatal human case, is highly lethal to ferrets and mice (13, 18). Sequence analysis of this virus's complete genome and that of nonlethal CH58 revealed amino acid differences encoded by the genes (13). Specifically, there are three amino acid differences in PB1 gene, four in PB2, four in PA, five in HA, one in NP, five in NA, one in M, and eight in NS. Here, we demonstrate the use of a model system in which recombinant viruses are generated by reverse genetics (RG) to study pathogenicity in ferrets and mice. This approach allowed us to distinguish whether high virulence of H5N1 VN1203 as compared with CH58 is caused by its surface glycoproteins HA and neuraminidase (NA), its NS gene, or its polymerase genes.

RESULTS

Model system for evaluation of pathogenicity in ferrets and mice

Human isolate VN1203 and chicken isolate CH58 are both lethal to chickens; however, only the human isolate is lethal to ferrets (13). To determine what gene segments contribute to VN1203 high virulence and systemic spread in ferrets and mice as compared with CH58, we used the eight-plasmid system (19). We constructed two eight-plasmid sets encoding individual genes of VN1203 and CH58, to generate RG VN1203 and CH58 recombinant viruses by DNA transfection. Sequence analysis showed that the RG viruses were identical to their respective parental viruses. To test the pathogenicity of the RG viruses, we intranasally inoculated ferrets with 10^6 50% egg infectious dose (EID_{50}) of virus. Infection with VN1203 virus was lethal to all three ferrets as early as 5 d after infection, but CH58 was not (Fig. 1 A). Ferret body temperatures were ~2°C higher after inoculation with VN1203 than CH58. Ferrets inoculated with VN1203 lost >20% of their body weight (Fig. 1 B), whereas those in the CH58 group maintained or increased their weight. These findings were similar to those observed after inoculation (a.i.) with the respective parental virus strains (13).

To determine the lethal dosage of VN1203 and CH58 in mice, we inoculated C57BL6 mice with different dilutions of the recombinant viruses. Mice inoculated with 10^3 EID_{50} of VN1203 showed considerable weight loss, and all died (Fig. 1, C and D). In contrast, mice inoculated with the same dose of CH58 virus showed no disease signs, even those infected with 10^6 EID_{50} of CH58 survived. Therefore, VN1203 was highly lethal and CH58 nonlethal in both mammalian models under these infection conditions.

Infection of Madin-Darby canine kidney (MDCK) cells with VN1203 resulted in large plaque formation (Fig. 1 E), and infection with CH58 resulted in smaller plaques. The VN1203 plaques had a mean size of 2.72 ± 0.17 mm. In contrast, plaques created by CH58 were on average 0.67 ± 0.17 mm which were significantly smaller than plaques of the human virus (Student's t test; $P < 0.001$).

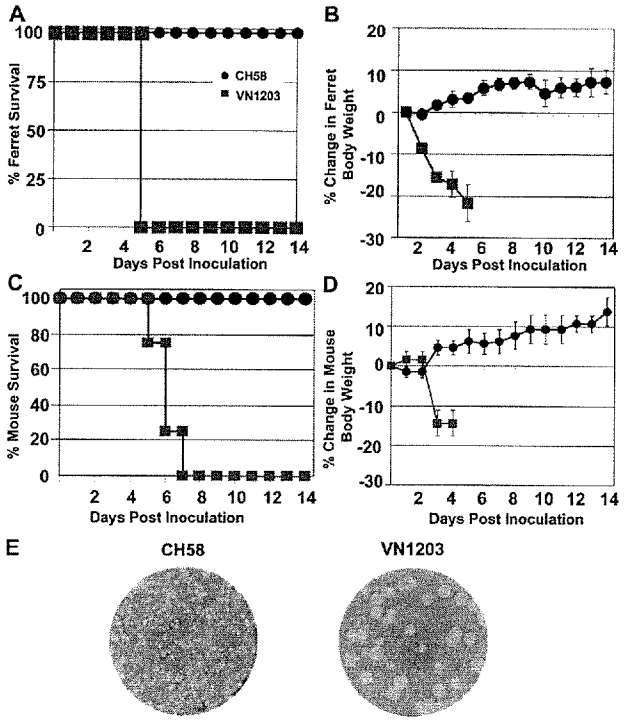

Figure 1. Effect of inoculation of ferrets and mice with VN1203 and CH58. (A) Survival rate of ferrets after intranasal inoculation with 10^6 EID_{50} of RG VN1203 ($n = 3$) or CH58 ($n = 3$) virus. (B) Mean ± SE percent weight change of groups of three ferrets after inoculation. (C) Survival rate after intranasal inoculation of groups of six mice with 10^3 EID_{50} of VN1203 or CH58. (D) Mean ± SE percent weight change of mice after inoculation. (E) Plaques on MDCK cells after titration of CH58 and VN1203 virus.

To monitor viral growth and spread, we titrated virus of ferret nasal wash specimens and organ homogenates. On days 3 and 5 a.i., virus titers were between 1 and 4 EID_{50}/ml logs higher in nasal washes from ferrets inoculated with VN1203 than in those inoculated with CH58 (Fig. 2 A). High virus titers were detected 4 d a.i. in brain, spleen, liver, and lung tissue homogenates from a ferret inoculated with VN1203 (Fig. 2 B).

Days 4 and 5 a.i. of ferrets with VN1203, histologic alterations were observed in the lungs, liver, and brain (Fig. 2 C). Lung peribronchiolar pneumonia was characterized by bronchiolar necrosis, hyperplasia, and intraluminal necrotic and inflammatory cells. Alveoli had hyperplasia and inflammatory cells. In the liver, there was hepatocellular vacuolization, biliary necrosis, and inflammatory cells. In the brain's neuropil, there was neuronal degeneration associated with inflammatory cells. Monocyte infiltrates were also in meninges and perivascular spaces. In contrast, the lungs, livers, and brains of ferrets inoculated with CH58 showed no apparent changes. In sum, the RG 1203 and CH58 viruses were identical to their respective parental viruses in genotype and phenotype.

BSL3+ isolators' space constraints allow testing of few animals per experiment. Therefore, we focused on reassortants

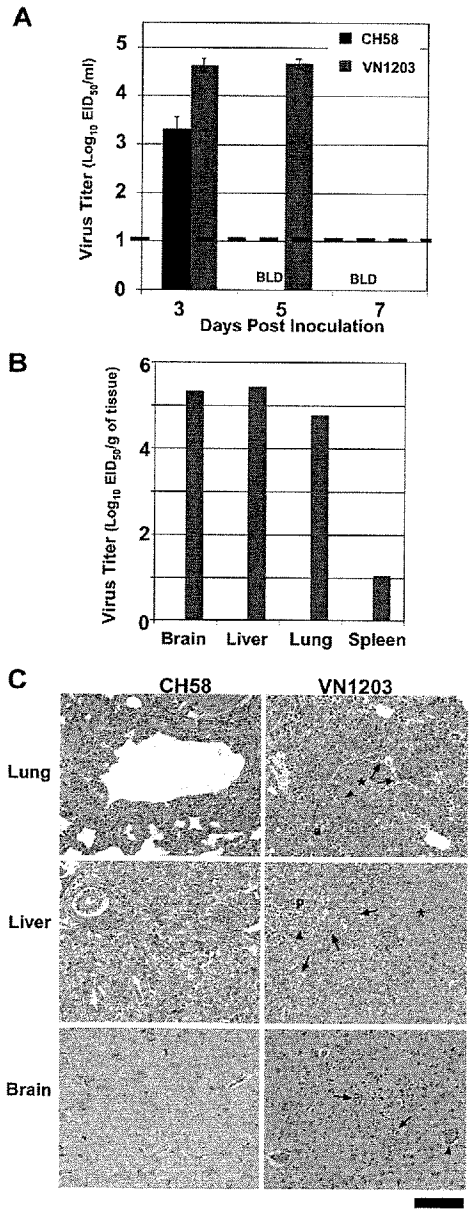

Figure 2. Virus titers and tissue pathology of RG VN1203 and CH58 H5N1 viruses in ferrets. (A) Mean virus titers ± in ferret nasal washes after VN1203 or CH58 inoculation. Dashed line indicates detection limit of 10^1 EID_{50}/ml. BLD, below level of detection. Error bars indicate that all animals deceased at this time point. (B) Viral titers in ferret organ homogenates 4 d a.i. (C) Hematoxylin and eosin–stained sections (20X) of ferret lung, liver, and brain 4–5 d a.i. All tissues from CH58 inoculation were normal. VN1203 ferret lung shows bronchiole (*) with epithelium loss (▲) and epithelial regeneration (↑), intraluminal necrotic and inflammatory cells in bronchioles and alveoli (A). Liver shows inflammatory cell infiltrate in parenchyma and portal tract (p) with blood vessels and biliary duct. Biliary epithelium necrosis (▲) and liver cells (↑). Normal liver cells (*) surround necrotic foci with inflammatory cells. Inflammatory cell infiltrate associated with degenerating and necrotic neurons (↑) and perivascular inflammatory cells (▲). Bar, 50 μm.

selected according to their virus life cycle function: surface glycoproteins, polymerase complex genes, and NS gene (Fig. 3). A previous systematic sequence comparison of all gene segments indicated that VN1203 and CH58 viruses differed most greatly in these genes (13).

Surface glycoprotein–reassortants do not alter pathogenicity

Systemic spread and high lethality of VN1203 in ferrets and mice could be caused by altered tissue tropism resulting from changes in HA and NA. VN1203 and CH58 differ by six amino acids in each surface glycoprotein (13). We therefore generated an RG VN1203-CH58(HA,NA) virus that had VN1203 six internal genes and CH58 HA and NA genes (Fig. 3). All ferrets inoculated with this recombinant virus died between days 6 and 11 a.i. (Fig. 4 A). All mice inoculated with VN1203-CH58(HA,NA) virus died 8 d a.i. (Fig. 4 B). Survival rate, weight loss, viral titers, and disease severity caused by VN1203-CH58(HA,NA) was similar to that of VN1203 in both models (Fig. 4).

10 days a.i. of ferrets with VN1203-CH58 (HA,NA), similar pathology as with VN1203 were observed in lungs, livers, and brains (Fig. 4 F). The lungs had bronchiolar necrosis, hyperplasia, and intraluminal necrotic and inflammatory cells (Fig. 4 F, top left). Within liver parenchyma, there was hemorrhage associated with necrotic and vacuolated hepatocytes (Fig. 4 F, top right). Brain alterations were consistent with a later infection stage. In addition, there was neuronal degeneration and gliosis (Fig. 4 F, bottom left). Moreover, the brain also had liquefactive necrosis (malacia) surrounded by foamy macrophages (gitter cells) and reactive astrocytes (Fig. 4 F, bottom right).

After titration on MDCK cells, VN1203-CH58(HA,NA) grew large plaques (2.87 ± 0.19 mm), as had VN1203 (Fig. 4 G). RG virus with surface glycoproteins of the human isolate and its remaining genes from the chicken isolate (CH58-VN1203(HA,NA)) grew small plaques (0.48 ± 0.17 mm). Therefore, plaque size in MDCK cells, lethality, and pathogenicity of these viruses in mice and ferrets were not affected by these sequence changes in HA and NA.

To determine whether VN1203 and CH58 viruses differed in their HA receptor specificity, we measured the viruses' affinity toward synthetic SA substrates p3′SL or p6′SL. In this assay, the lower the dissociation constant (K_d [μM of sialic acid]), the more strongly virus binds to the receptor. VN1203 had a K_d of 1.50 ± 0.21 to 3′SL but a K_d > 100 to 6′SL. Therefore, VN1203 has a greater affinity for α-(2,3)–linked than α-(2,6)–linked SA receptors. Similarly, CH58 had a K_d of 1.10 ± 0.10 toward 3′SL and a K_d > 100 for 6′SL. Thus, both VN1203 and CH58 have preferential binding affinity for α-(2,3)–linked SA receptors, typical of avian viruses.

Contribution of NS gene to lethality of VN1203 in ferrets but not mice

Many studies have established that NS1 regulates innate immunity by modulating the host type I interferon response, which has both antiviral and immunoregulatory functions.

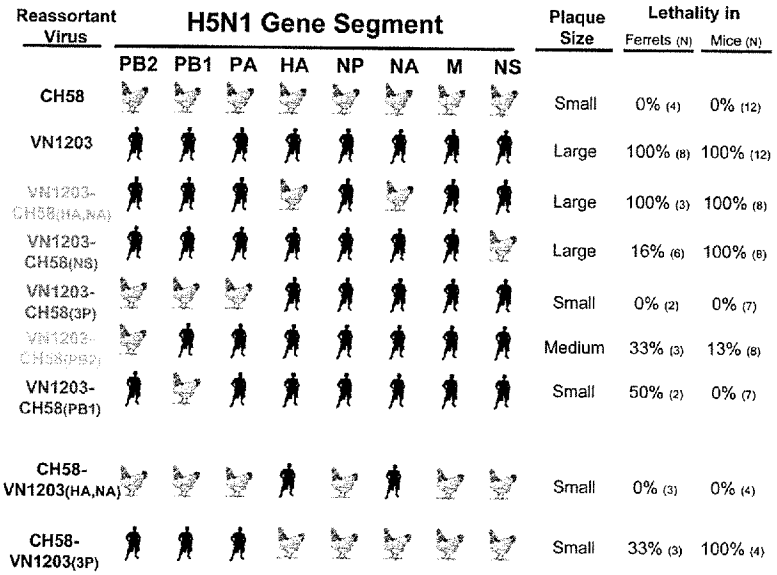

Figure 3. Generation of reassortant viruses with summary of plaque morphology and lethality. Human and chicken symbols denote source of gene segment (human VN1203 isolate or chicken CH58 isolate). The colors of the names of the RG reassortant viruses correspond to the colors in all data graphs of animals infected with those viruses. Mean plaque size was categorized as small (<1 mm), medium (1–2 mm), or large (>2 mm). The percent lethality caused by each RG virus and total number (N) of ferrets or mice used is indicated.

We therefore generated single-gene reassortants that combined CH58 NS with the remaining seven VN1203 genes. Five out of the six ferrets inoculated with VN1203-CH58(NS) virus survived (Fig. 5 A). They demonstrated 20% less weight loss than did ferrets inoculated with VN1203 (Fig. 5 B). All mice inoculated with single-reassortant VN1203-CH58(NS) lost weight and died by day 8 a.i. (Fig. 5, C and D). Thus, NS–reassortants show differences in lethality between ferrets and inbred mice.

Polymerase complex contribute to lethality of VN1203 in ferrets and mice

11 amino acid differences in polymerase genes (4 in PB2, 3 in PB1, and 4 in PA) distinguish VN1203 and CH58. Inoculation with reassortant virus VN1203-CH58(3P)—consisting of CH58 PB2, PB1, and PA genes and the remaining genes from VN1203—was not lethal to ferrets and mice (Fig. 6, A and C). Animals inoculated with VN1203-CH58(3P) showed no weight loss (Fig. 6, B and D). As with CH58, VN1203-CH58(3P) virus generated small plaques (0.88 ± 0.50 mm), on MDCK cells (Fig. 6 E). Thus, attenuation and reduction in plaque size, compared with VN1203 plaques, is mediated by polymerase complex genes.

Ferrets inoculated with single-gene reassortant VN1203-CH58(PB2) experienced modest weight loss, and two out of three ferrets survived (Fig. 6, A and B). Ferret nasal viral titers were 2 and 1 log lower on days 3 and 5 a.i., respectively, in those inoculated with chicken PB2 reassortants. Mice inoculated with VN1203-CH58(PB2) showed no weight loss, and one of eight mice died 10 d a.i. (Fig. 6, C and D). The generation of a RG virus of VN1203 with a point mutation in PB2 changing lysine (K) at position 627 to glutamic acid (E) was not lethal when administered to mice (unpublished data). This demonstrates that K^{627} of VN1203 PB2 contributes to lethality.

Ferret inoculation with single-gene reassortant VN1203-CH58(PB1) resulted in 50% survival (Fig. 6 A). Infection of mice with the same reassortant resulted in 15% weight loss 7 d a.i. However, all mice gained weight after day 9 and survived (Fig. 6, C and D). Interestingly, this single-gene CH58(PB1) reassortant also grew significantly smaller plaques (0.29 ± 0.04 mm), than VN1203 on MDCK cells (Fig. 6 E) (Student's *t* test, $P < 0.001$). These results underscore the importance of genetic changes in PB2 and PB1 to VN1203 replication efficiency. Collectively, these results demonstrate that the polymerase genes contribute to pathogenicity in both mice and ferrets.

To determine whether the three VN1203 P-genes are sufficient for high lethality, we generated a reassortant virus comprising the human isolate's polymerase genes and the remaining five segments from the chicken isolate. Inoculation of mice with CH58-VN1203(3P) resulted in severe loss of body weight and 100% lethality between days 9 and 11 a.i. Two of three ferrets inoculated with this CH58-VN1203(3P) virus survived and weight loss was similar to those infected with VN1203-CH58(NS) (Fig. 5). Thus, the three human P-genes are sufficient to convert a nonlethal virus into a lethal virus in mice but not in ferrets.

VN1203 polymerase complex possesses significantly higher transcription/replication activity

To assay polymerase activity of VN1203 and CH58, we transfected 293T cells with plasmids containing their PB2,

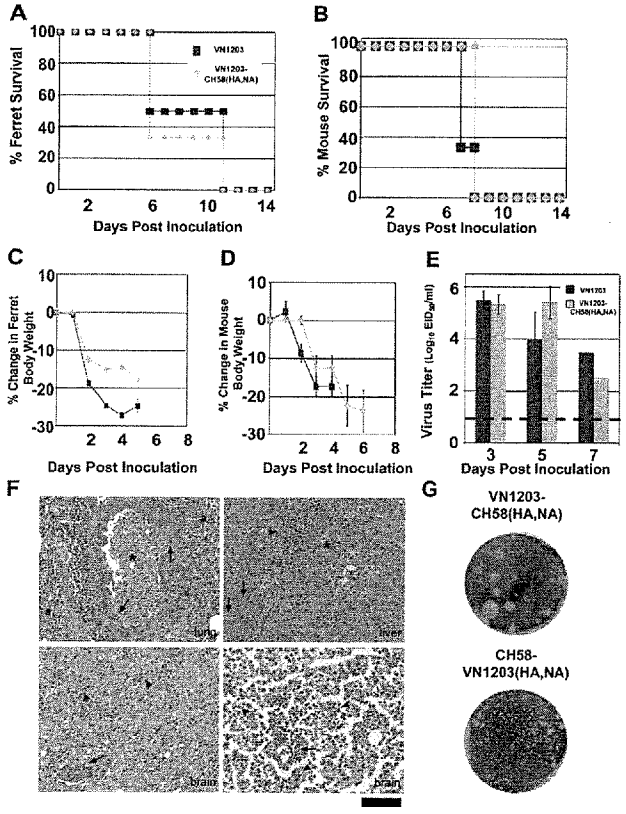

Figure 4. Reassortment of HA and NA does not affect lethality or pathogenicity in vivo or in cell culture. Survival (A) of ferrets and (B) mice a.i. with VN1203 (n = 2 ferrets, 8 mice) or VN1203-CH58(HA,NA) (n = 3 ferrets, 8 mice). Mean weight change (C) of ferrets and (D) mice ± SE. (E) Mean viral titers from ferret nasal washes ± SE. Dashed line indicates detection limit of 10^1 EID_{50}/ml. (F) Hematoxylin and eosin-stained sections (20X) of ferret lung, liver and brain 10 d a.i. with VN1203-CH58(HA,NA). Lung (top left) shows bronchiole (*) with epithelial hyperplasia (↑), necrotic debris in lumen and inflammatory cells in alveoli (A). Liver (top right) shows disrupted parenchyma with vacuolated hepatocytes associated with hemorrhage (*). Monocytes (▲) surround parenchyma and hyperplastic biliary cells (↑) extend into parenchyma. Brain (bottom left) shows perivascular inflammatory cells (↑), hypercellular neuropil with capillary endothelial hyperplasia (▲), gliosis and monocytes. Brain region (bottom right) with liquefactive necrosis and foamy macrophages (gitter cells, ▲) and perivascular monocytes (↑). Bar, 40 μm. (G) Plaque formation after virus titration on MDCK cells.

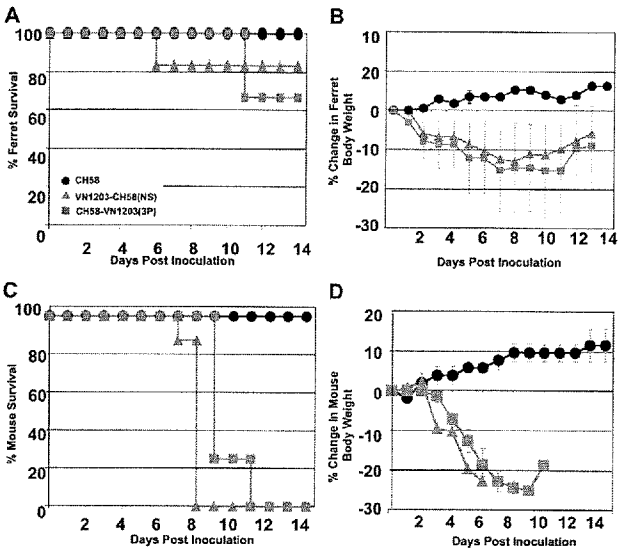

Figure 5. NS genes contribute to lethality of VN1203 in ferrets but not in mice. Survival rate (A), and mean weight change ± SE (B) of ferrets inoculated with CH58 (n = 3), VN1203-CH58(NS) (n = 6), or CH58-VN1203(3P) (n = 3) reassortant virus. Survival rate (C) and mean weight change ± SE (D) of mice (n = 8) inoculated with CH58 (n = 8), VN1203-CH58(NS) (n = 8), or CH58-VN1203(3P) (n = 4).

PB1, PA, and NP genes and a luciferase reporter plasmid. Because cDNA encoding luciferase is controlled by the influenza A virus M segment's noncoding region, luciferase levels reflected overall transcription and replication activity of the polymerase complex (20). Luciferase activity in human embryonic kidney 293T cells cotransfected with plasmids containing VN1203 polymerase complex and NP had luciferase activity (relative light units; RLUs) 3.5-fold that of cells cotransfected with CH58 polymerase complex and NP (Student's t test; $P < 0.01$) (Fig. 6 F). Therefore, the VN1203 polymerase complex possessed significantly higher transcription/replication activity than the CH58 polymerase complex.

To compare polymerase activity of VN1203 PB2 and PB1 segments, we replaced the individual PB2 and PB1 segments of VN1203 with those of CH58 (Fig. 6 F). When VN1203-PB2 was replaced with CH58-PB2, luciferase activity was only 22% of VN1203. When VN1203-PB1 was replaced with CH58-PB1, luciferase activity was 58% of VN1203 (Fig. 6 F). The effect of CH58 NP was assayed by cotransfecting VN1203 PB2, PB1, and PA plasmids with the CH58 NP plasmid. CH58 NP did not decrease the high transcription/replication activity of the VN1203 polymerase as there was no notable difference in luciferase activity as compared with the transfection of VN1203 PB2, PB1, PA and NP plasmids (unpublished data). Therefore, both CH58 PB2 and PB1 reduced the VN1203 transcription/replication activity.

DISCUSSION

Here, we have demonstrated the use of a model system that uses RG to map some factors that contribute to high virulence of a 2004 H5N1 influenza virus isolate. We showed that inoculation of ferrets with recombinant human influenza virus isolate VN1203, unlike inoculation with CH58, is followed by rapid systemic spread to the brain and liver, resulting in high lethality. Survival of all mice and ferrets inoculated with a RG VN1203 virus that had its polymerase genes derived from the chicken isolate showed that these genes are important for virulence. In the reverse experiment using the chicken-isolate backbone with the VN1203 polymerase genes, all inoculated mice died. Therefore, polymerase subunits

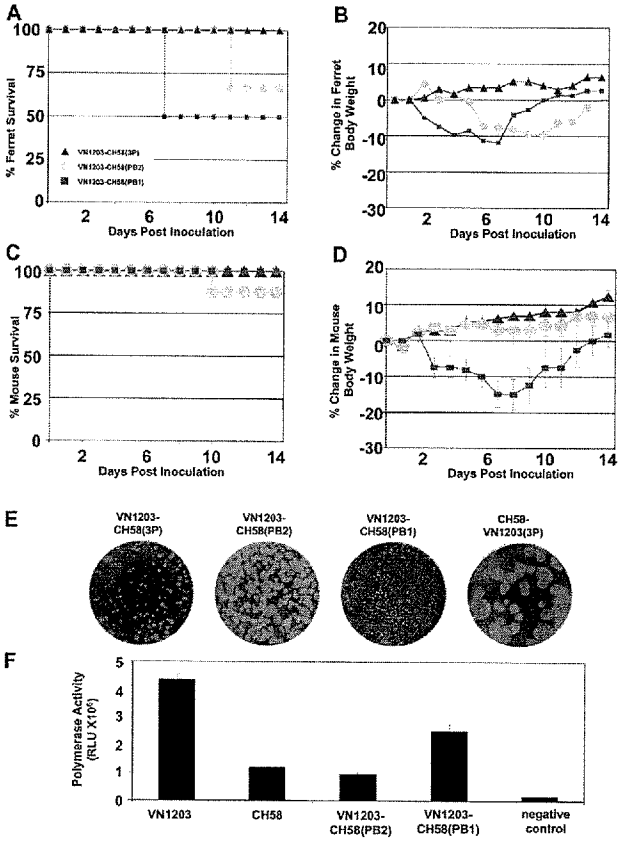

Figure 6. Polymerase complex genes contribute to lethality of VN1203. Survival rate (A) and mean weight change ± SE (B) of ferrets inoculated with polymerase gene-reassortant viruses. Survival rate (C) and mean weight change ± SE (D) of mice inoculated with polymerase gene-reassortant viruses. (E) Plaque formation in MDCK cells after virus titration. (F) Polymerase activity assayed by viral UTR-driven luciferase reporter gene. 293T cells transfected with plasmids containing VN1203 or CH58 PB2, PB1, PA, and NP genes plus a luciferase reporter plasmid, or with only VN1203 NP and the reporter plasmid (negative control). After 24 h, luciferase activity was assayed in cell extracts. Results are mean ± SE of triplicate transfections.

PB2, PB1, and PA are central to influenza's replication cycle and required for viral RNA replication and transcription (21). The NH_2-terminal region of PB1 interacts with the COOH-terminal region of PA, whereas the COOH-terminal region of PB1 interacts with the NH_2 terminus of PB2 (22). K^{627} of PB2 was previously demonstrated to be required for 1997 H5N1 viruses' high virulence in mice (16, 23), which was consistent with our finding that the human virus isolate whose PB2 contained K^{627} was more lethal than that with E^{627}.

Despite clear evidence of K^{627} role in the virulence and host range restriction (24), some lethal 1997 and 2004 H5N1 isolates, such as A/quail/Vietnam/36/04 (H5N1) viruses, have avian-like residue E^{627}, indicating the importance of other genes or of the other three amino acids in PB2. Indeed, the attenuation of PB1-reassortants indicated that PB1 contributes significantly to this effect. The same three amino acids of VN1203 changes in PB1 are found in the isolate A/quail/Vietnam/36/04 (H5N1), which is lethal to ferrets (13). Viral-like reporter replication was more efficient when PB1 is derived from an avian virus (25).

It was difficult to conclusively determine the contributions of single P genes to pathogenicity because of the small number of ferrets used. However, different growth properties observed in three different mammalian species (a canine cell line, mice, and ferrets) and the mini-genome assay in human cells suggest a general, not species-specific, enhancement of replication. Collectively, these results suggest that before and/or after transmission from chickens to humans, adaptive changes appear in PB2 and PB1 that result in better interaction of polymerase subunits and/or between polymerase subunits and host factors, leading to more rapid generation of infectious virus particles. It is possible that rapid growth is prerequisite for systemic spread after the initial infection in small animal models. The lack of evidence for systemic spread in humans indicates that efficient growth in yet to be identified cell types may contribute to severe disease and death in humans.

HA's role in virus cell binding and entry implies it is important for efficient virus spread after infection (26). The presence of HA's multibasic cleavage site is well correlated with influenza A viruses' high pathogenicity (12). This sequence results in the cleavage of HA by intracellular furin-like proteases (27). In this study, both the chicken and human isolate have polybasic amino acids in the connecting HA peptide which is sufficient for rapid systemic viral spread, resulting in death of chickens (28) and is essential for the human 1997 H5N1 isolates' virulence in mice (16). However, contributions of HA receptor-binding properties to cell tropism and virus transmission are not known. Our finding that recombinant human virus containing the chicken HA retained its lethality demonstrates that HA residues outside the cleavage site do not have a critical impact on tropism. Thus, VN1203 (H5N1) influenza's systemic spread and neurotropism are not the result of changes in receptor binding. H5N1 viruses whose HA is selective for α-(2,3)–linked SA can spread efficiently after experimental infection. It is noteworthy that CH58 and VN1203 and most recent H5N1 viruses have S^{223} in their HA and that this residue located in the 220-loop of the HA is critical for receptor specificity to α-(2,3)–SA (29). Substitution of S^{223} to N^{223} has been shown to alter receptor specificity to both avian-like α-(2,3)–linkage and humanlike α-(2,6)–linkage (29). Inefficient binding and cell entry of an avian H5N1 virus in a mammalian host can be compensated by intracellular cleavage of the HA and by efficient replication/transcription of the viral genome by PB1 or PB2. Thus, multibasic amino acids in the cleavage site of the HA are necessary but not sufficient for high lethality in mammalian species.

Viral pneumonia with acute respiratory distress syndrome is the main cause of rapid death in human cases of H5N1 influenza infection (7). H5N1/97 viruses induce much higher

transcription of proinflammatory cytokines in human primary monocyte-derived macrophages in vitro, particularly TNF-α and interferon-β, than do H3N2 human influenza viruses (30). The nonstructural (NS) gene-segment of H5N1/97 viruses contributed to the increase in TNF-α induced by the virus. Although PB1 and PB2 roles appear to be independent of the host and cell type, different effects of genetic changes in the CH58-NS segment in ferrets and mice suggest differences in the host factors regulated by NS1. NS1 is known to have the potential to inhibit host type I interferon responses to virus (31, 32). It is possible that ferrets and feral mice express functional interferon-inducible antiviral Mx protein, whereas many inbred strains, such as the C57BL6 mice in these studies, do not (33, 34). Mx has been shown to specifically inhibit influenza A (35, 36). Mice expressing Mx could be used to determine whether observed differences in the role of NS1 reflect an experimental limitation, such as dependence of lethality on virus dose, or indeed reflect species-specific interaction with the innate immune system. There is one amino acid difference between the NS2/NEP protein of VN1203 and CH58, which is unlikely to play a role in the differences in pathogenesis. However, as to whether the 10–amino acid deletion at the COOH terminus of VN1203 and/or specific amino acids in the NS1 protein are responsible for differences in pathogenesis should be further investigated.

Our results suggest that antivirals directed against the polymerase proteins could be developed. The mini-genome assay we established in this study for highly pathogenic H5N1 viruses can perhaps be used in high-throughput screening. Inhibitors that reduce polymerase activity about two- to threefold in cell culture–based assays are candidates for therapeutic testing in the mouse or ferret model. These new types of antivirals could complement the use of NA inhibitors and the M2-ion channel blocker amantadine.

Although H5N1 viruses such as VN1203 may be too lethal to cause a pandemic, our results demonstrate that changes in only the polymerase genes of an H5N1 influenza virus are sufficient to dramatically alter its pathogenic potential. Even changes in single segments PB2 and/or PB1 attenuated the human virus isolate's pathogenicity. 1957 and 1968 influenza pandemics were caused by replacement of the PB1 gene with avian PB1 (37). All eight virus segments causing the 1918 pandemic originated from an avian host (38). Thus, three pandemic viruses had avian-like PB1 genes that may be important to high replication efficiency, as we have demonstrated here with VN1203. Isolation of PB2 K^{627} in migrating birds (39, 40) is cause for concern because it appears to be a critical step for adaptation to a mammalian host. Those viruses may acquire additional changes in PB2, PB1 and HA resulting in the ability to spread from human to human.

MATERIALS AND METHODS

Generation of recombinant viruses by RG. A/Vietnam/1203/04 (H5N1) and A/chicken/Vietnam/C58/04 (H5N1) influenza viruses were obtained from the World Health Organization collaborating laboratories and propagated in allantoic cavities of 10-d-old embryonated chicken eggs. Experiments were conducted in biosafety level 3+ (BSL3+) conditions approved for work with these viruses. RT-PCR was used to amplify all eight viral genes, and viral cDNAs were inserted into dual-promoter plasmid pHW2000 (19). All plasmids were sequenced, and QuikChange Site-Directed Mutagenesis kits (Stratagene) were used to generate coding sequences in plasmids identical to PCR fragment sequences. Recombinant viruses were generated by DNA transfection of MDCK/293 T cells. Transfection supernatant was injected into 10-d-old embryonated chicken eggs, and virus stock prepared, sequenced and titrated.

Sequence analysis. Viral RNA isolated directly from virus-containing allantoic fluid using RNA isolation kit (RNeasy; QIAGEN). The universal primer set for influenza A virus was used for RT-PCR (41). Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children's Research Hospital determined DNA template sequence using Big Dye Terminator (v.3) chemistry and synthetic oligonucleotides. Samples were analyzed on 3700 DNA analyzers (Applied Biosystems).

Inoculation of ferrets. Male ferrets, (Marshall's Farms) aged 3–5 mo and seronegative by HA inhibition test for exposure to circulating influenza B and H1N1, H3N2, and H5N1 influenza A viruses, were used. Ferrets were anesthetized with isoflurane and inoculated intranasally with 10^6 EID_{50} of infectious virus. Clinical signs of infection, weight, and temperature were recorded daily. Body temperature was measured using subcutaneous implantable temperature transponders. All animals impaired in their ability to eat or drink were killed. All animal studies were conducted under applicable laws and guidelines and after approval by St. Jude Children's Research Hospital animal care and use committee.

Titration of virus in ferret upper respiratory tract. On days 3, 5, and 7 after inoculation, ferrets were anesthetized with intramuscularly injected ketamine (25 mg/kg), and 0.5 ml of sterile PBS was introduced into each nostril and collected in containers. Virus was titrated in 10-d-old embryonated chicken eggs and expressed as $\log_{10}EID_{50}$/ml. Limit of detection was 1 $\log_{10}EID_{50}$/ml.

Virus titration and histopathologic analysis of ferret organs. Lung, brain, spleen, and liver were collected 4 d a.i. or at time of death. Samples were weighed and homogenized in PBS. Homogenates were titrated in 10-d-old embryonated chicken eggs to determine $\log_{10}EID_{50}$ per tissue gram. Samples were fixed in 10% buffered formalin, paraffin embedded, cut into 5-μm sections, mounted on slides, stained with hematoxylin and eosin, and examined by light microscopy.

Inoculation of mice. 7-wk-old C57BL6 female mice (The Jackson Laboratory) were lightly anesthetized with isoflurane and inoculated intranasally with 10^3 EID_{50} of infectious virus in 50 μl of PBS. Weight, clinical signs of infection, and survival were recorded daily.

Plaque assay. Confluent monolayers of MDCK cells were inoculated with 10-fold dilutions of influenza virus and incubated at 37°C for 1 h. The inoculum was removed, and cells were washed and overlaid with MEM containing 1% agarose and 0.2% serum albumin. After 3 d at 37°C, cells were stained with 0.1% crystal violet in 37% formaldehyde solution and plaque morphology evaluated. Plaque size was measured using fine scale magnifying comparator (6X). The probability of a significant difference in plaque size between viruses was computed using a Student's paired *t* test, with a two-tailed distribution.

Assay of virus binding to sialic acid–containing substrates. Virus binding to fetuin was measured by direct solid-phase assay with immobilized virus and horseradish peroxidase-conjugated fetuin (42, 43). Affinity to 3′- and 6′-sialylglycopolymer obtained by conjugation of 1-*N*-glycyl derivative of 3′-sialyllactose (p3′SL) or 6′-sialyllactose (p6′SL) was measured in competitive assay based on inhibition of peroxidase-labeled fetuin binding (44). Sialoglycopolymers were gifts from N. Bovin (Shemyakin Institute of

Bioorganic Chemistry, Moscow, Russia). The dissociation constant (K_d) of virus–receptor analogue complex was calculated for each virus as mean of four experiments.

Luciferase assays. 293T cells subconfluent monolayers (7.5×10^5 cells in 35-mm dishes) were transfected (Mirus Bio) with 2 μg of luciferase reporter plasmid (EGFP open reading frame in pHW72-EGFP substituted with luciferase gene [reference 20]) and mix of PB2, PB1, PA, and NP plasmids of VN1203 and CH58 viruses in quantities of 1, 1, 1, and 2 μg, respectively. After 24 h, cell extracts that were prepared in 500 μl of lysis buffer and luciferase levels were assayed with Luciferase Assay System (Promega) and BD Monolight 3010 luminometer (BD Biosciences). Experiments were performed in triplicate.

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The RNA Polymerase PB2 Subunit Is Not Required for Replication of the Influenza Virus Genome but Is Involved in Capped mRNA Synthesis

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An established cell line, clone 64, in which the expression of the RNA polymerase PB1 and PA subunit genes and the nucleoprotein (NP) gene but not the PB2 subunit gene of influenza virus can be induced by the addition of dexamethasone, was used to analyze the replication and transcription machineries of the influenza virus. Both NS-CATc and NS-CATv, the chimeric nonstructural protein chloramphenicol acetyltransferase (NS-CAT) RNAs in the sense and antisense orientations positioned between the 5′- and 3′-terminal sequences of influenza virus RNA segment 8 (the NS gene), respectively, can be transcribed into the corresponding complementary-strand RNA in clone 64 cells only when treated with dexamethasone. Although sense-strand poly(A)$^+$ CAT RNA was detected in the dexamethasone-treated clone 64 cells transfected with NS-CATv RNA, CAT activity was not detected in these cells and the isolated poly(A)$^+$ CAT RNA was inert in an in vitro translation system. However, when the poly(A)$^+$ CAT RNA was capped by using a purified yeast mRNA capping enzyme (mRNA guanylyltransferase), the capped poly(A)$^+$ CAT RNA became translatable in the in vitro translation system. These results indicated that PB1, PA, and NP can support the replication of the influenza virus genome as well as the transcription to yield uncapped poly(A)$^+$ RNA and that PB2 is specifically required for the synthesis of capped RNA.

The influenza A virus has a genome consisting of eight single-stranded RNA segments of negative polarity, altogether encoding at least 10 viral proteins (reviewed in reference 21). Replication and transcription of the influenza virus genome are catalyzed by a virus-encoded RNA-dependent RNA polymerase (reviewed in references 14 and 21). In virions, the ribonucleoprotein cores are composed of genomic RNA, (vRNA), RNA polymerase, and nucleoprotein (NP). The RNA polymerase is composed of three subunits, PB1, PB2, and PA (reviewed in references 14 and 20), which are tightly associated at the double-stranded stem region of the panhandle formed by the 5′ and 3′ termini of each RNA segment (10, 12). RNA polymerase plays an essential role in both replication and transcription.

In virus-infected cell nuclei, three modes of RNA synthesis occur (reviewed in references 20 and 22): (i) transcription of the vRNA into mRNA which contains a cap structure at the 5′ terminus and a poly(A) tail at the 3′ terminus, (ii) the vRNA-directed synthesis of a full-length cRNA without any modifications (the first step of replication), and (iii) the cRNA-directed synthesis of vRNA (the second step of replication). For transcription, cellular RNA with the cap-1 structure is used as a primer (reviewed in reference 20). Biochemical and genetic analyses have revealed that PB1 is involved in transcription initiation and RNA chain elongation, while PB2 recognizes and binds to the cap-1 structure of the cellular mRNA (reviewed in reference 20). PA is present in the RNA polymerase complex together with PB1 and PB2, but its function has not been elucidated. NP, a major component of the ribonucleoprotein cores, is associated at every 15 to 20 nucleotides of the RNA segments (4, 41), and it is required for the efficient elongation of RNA chains (11).

In contrast, little is known about the molecular mechanism of replication. It is believed without conclusive evidence that replication requires all three subunits of RNA polymerase and NP and that it occurs in a primer-independent manner (reviewed in reference 20). In replication systems in vitro using crude extracts prepared from virus-infected cells, the first step of replication has been studied (2, 8, 36). Furthermore, a low level of the second step of replication has been detected by the use of fractionated nuclear extracts (24, 33). However, since all these systems monitored RNA synthesis directed by endogenous templates, the de novo initiation of RNA synthesis and the elongation of RNA chains initiated in infected cells were indistinguishable.

Recently, we developed the clone 76 cell line, in which all three RNA polymerase genes and the NP gene are integrated in the chromosome and can be expressed in response to dexamethasone (17, 18, 28). Exogenously added model RNAs such as chloramphenicol acetyltransferase (CAT) RNAs in the sense and antisense orientations, positioned between the 5′- and 3′-terminal sequences of segment 8 RNA encoding nonstructural (NS) proteins, can be replicated and transcribed in clone 76 cells incubated with dexamethasone. In an effort to elucidate the function of each polymerase subunit protein, we established another cell line, clone 64, which carries the PB1 and PA subunit genes of RNA polymerase and the NP gene but lacks the PB2 gene (28). In this study, chimeric NS-CAT RNAs in the sense (NS-CATc) and antisense (NS-CATv) orientations were replicated in dexamethasone-treated clone 64 cells. However, CAT activity was undetectable in NS-CATv

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RNA-transfected clone 64 cells. In addition, we describe how the poly(A)$^+$ CAT RNA isolated from clone 64 cells incubated with dexamethasone lacks a cap structure at the 5′ end and how it cannot be translated in vitro with a rabbit reticulocyte system. After adding the cap-1 structure, the poly(A)$^+$ CAT RNA was translated into CAT protein. These results indicated that the PB1 and PA subunits of RNA polymerase and NP are sufficient for replication of the influenza virus genome and for transcription to yield uncapped poly(A)$^+$ RNA. The possible involvement of PB2 in cap recognition is discussed.

MATERIALS AND METHODS

Cells. Mouse C127 cells and derivative cell lines, clones 64 and 76 (28), were incubated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

RT-PCR. Monolayer cultures of clones 64 and 76 at 40 to 50% confluence were incubated with 10^{-6} M dexamethasone for 6 h at 37°C, and total cellular RNA was extracted with acid guanidinium thiocyanate–phenol-chloroform (3). Twenty micrograms of total cellular RNA was amplified by reverse transcriptase (RT)-mediated PCR (39) with the synthetic oligonucleotides PB2-SN3 (5′-TACCATGGCCACAATTATTGCTTCG-3′) and PB2-SN1 (5′-ATGGAAAG AATAAAAGAACTAAGAAATC-3′), which correspond to positions 1236 to 1212 and 28 to 55 in the sense orientation of the PB2 gene, respectively, to detect PB2 mRNA. Also, PB1-SN5 (5′-CTGTCGACTCCGGCTTGAATCCCTT-3′) and PB1-SN4 (5′-GATGTCAATCCGACCTTACTTTTCT-3′), which correspond to positions 1418 to 1394 and 28 to 52 in the sense orientation of the PB1 gene, respectively, were used for RT-PCR to detect PB1 mRNA.

Immunofluorescence. Monolayer cultures at 40 to 50% confluence grown on coverslips were incubated with 10^{-6} M dexamethasone for 17 h at 37°C. The cells were washed with phosphate-buffered saline (PBS) and fixed with 2% formaldehyde–PBS at room temperature for 5 min. The cells were incubated with 100 μl of rabbit anti-PB1, anti-PB2, anti-PA (1), or anti-NP antibody at a 1:200 or 1:1,000 dilution for 30 min at room temperature, were washed three times with 0.1% Nonidet P-40–PBS, and were stained with fluorescein isothiocyanate-conjugated goat F(ab′)$_2$ anti-rabbit immunoglobulin G (H + L; Tago) at a 1:200 dilution at room temperature for 30 min. After a washing with 0.1% Nonidet P-40–PBS, the cells were mounted in 100 mM Tris-HCl (pH 8.0)–90% glycerol–1 μg of paraphenylenediamine per ml for observation under a fluorescence microscope (Olympus).

In vitro RNA synthesis. Plasmids pOUMS101 (40) and pT7/NS-CATc (the same as pT7/NS-CATm described in reference 17) were cleaved with *Mbo*II, and the T7 polymerase reaction was carried out by the standard protocol (5) with RNasin (Takara Shuzo) used as a supplement in the presence or absence of [α-^{32}P]UTP (Amersham). Template DNA was removed by DNase I (Takara Shuzo) digestion. These RNA transcripts were used after phenol-chloroform extraction.

RNA transfection. For RNA transfection, 150 ng (in 60-mm-diameter dishes for CAT assay) or 100 to 500 ng (in 100-mm-diameter dishes for Northern [RNA] hybridization) of RNA was mixed with 18 μg (for CAT assay) or 36 μg (for Northern blotting) of lipofection reagent {*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium-methylsulfate; DOTAP, Boehringer Mannheim}. The mixture was incubated at room temperature for 15 min; then 2.4 ml (for CAT assay) or 4.8 ml (for Northern blotting) of Dulbecco's modified Eagle's medium containing 0.21% bovine albumin was added. Cells at 40 to 50% confluence were incubated with 10^{-6} M dexamethasone for 24 h at 37°C before transfection with the RNA-DOTAP complexes described above.

Northern hybridization. Cells (in 100-mm dishes) were incubated with 10^{-6} M dexamethasone for 24 h before transfection with 150 ng of the RNA-DOTAP complexes. Six hours later, total cellular RNA was prepared as described above. Poly(A)$^+$ RNA was purified from total cellular RNA by using oligo(dT)$_{30}$-Latex (Takara Shuzo). Total cellular RNA or poly(A)$^+$ RNA was resolved by electrophoresis on 1% formaldehyde-agarose gels and transferred to nylon membranes (23). Northern hybridization was performed with the ^{32}P-labeled synthetic oligonucleotide probes, SN24 (5′-AGTAGAAACAAGGGTGTT-3′, not found in the mRNA sequence) to detect cRNA, SN25 (5′-AGCAAAAGCAGGGT GAC-3′) for vRNA, and the 0.7-kb *Bam*HI CAT fragment from pOUMS101 (40) for mRNA as described previously (32, 38).

CAT assay. Cells (in 60-mm dishes) were incubated with 10^{-6} M dexamethasone for 24 h before transfection with 150 ng of the RNA-DOTAP complexes. Six hours later, 2.5 ml of Dulbecco's modified Eagle's medium containing 0.21% bovine albumin was added and the cells were cultivated for 18 h in the absence of dexamethasone. The medium was changed to fresh Dulbecco's modified Eagle's medium containing 10% fetal calf serum, and the cells were incubated for a further 9 h. Cells were harvested at various times after transfection, and the CAT assay proceeded as described by Gorman et al. (9). About 100 μg of protein was assayed.

In vitro translation. In vitro translation was performed according to the manufacturer's (Amersham) recommendations, using 0.2 μg of poly(A)$^+$ RNAs from clones 64 and 76, as well as from the parental C127 cells described above, or 2 μg of poly(A)$^+$ RNA which was capped in vitro as described below plus L-[^{35}S]methionine and rabbit reticulocyte lysate. Fifty microliters of the reaction mixture was incubated for 1 h at 30°C and for another 10 min at 37°C. After addition of lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA, 0.5% deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 1 mM phenylmethylsulfonyl fluoride, 1 μg of leupeptin per ml), the cell lysate was centrifuged for 30 min at 15,000 rpm in an Eppendorf centrifuge. The supernatant was incubated first with an anti-CAT antibody (5 Prime → 3 Prime Inc.) for 8 h at 4°C and then with protein A-agarose (Pharmacia) for 15 min at room temperature. Precipitates were loaded onto a 7.5 to 15% gradient SDS-polyacrylamide gel and analyzed with a Fujix BAS2000 (Fuji Film Inc.).

Addition of the cap structure. The capping reaction proceeded essentially as described previously (14). Four micrograms each of poly(A)$^+$ RNAs from clone 64 and 76 cells was incubated with 5 U of the mRNA capping enzyme purified from yeast cells (14) in the presence of [α-^{32}P]GTP (Amersham). After phenol-chloroform extraction and ethanol precipitation, the poly(A)$^+$ RNAs were loaded onto a 3% polyacrylamide gel and exposed to Fuji RX X-ray film.

For in vitro translation, poly(A)$^+$ RNAs were capped and methylated with 5 U of mRNA capping enzyme prepared from vaccinia virus (37) in the presence of 50 μM *S*-adenosyl-L-methionine (Boehringer Mannheim) and GTP. After phenol-chloroform extraction and ethanol precipitation, the poly(A)$^+$ RNAs were used in the in vitro translation system described above.

RESULTS

Neither the PB2 gene transcript nor PB2 protein was detectable in clone 64 cells. We established two cell lines, clones 64 and 76, in which the expression of the RNA polymerase genes and the NP gene of influenza virus can be induced in response to dexamethasone by transfection into mouse C127 cells with the hormone-inducible expression plasmids pBMSA-PB1, pBMSA-PB2, pBMSA-PA, and pBMSA-NP (28). In cultured cells of clone 64 (a mouse cell line carrying cDNAs for influenza virus RNA segments 2, 3, and 5 [28]), the PB1 and PA subunit genes of RNA polymerase and the NP gene are expressed in response to dexamethasone. On the other hand, the PB2 subunit gene is undetectable in both chromosomal and extrachromosomal fractions of clone 64 cells by Southern hybridization (28). By RT-PCR, the PB2 gene transcript was detected in dexamethasone-treated clone 76 cells (a mouse cell line carrying cDNAs for RNA segments 1, 2, 3, and 5 [28]) but not in clone 64 cells (Fig. 1, lanes 3 and 5), whereas the PB1 gene transcript was found in both clone 64 and clone 76 cells (lanes 2 and 4). Furthermore, in PCR analysis of the genomic DNA, the PB2 gene was undetectable in clone 64 cells but it was present in clone 76 cells (data not shown). We performed RT-PCR and PCR under conditions that allowed mRNA and DNA, respectively, to be detected at a level of one copy per cell. In good agreement with the RNA and DNA analyses, PB2 protein was undetectable in dexamethasone-treated clone 64 cells, but it was revealed in both the nucleus and cytoplasm of clone 76 cells by indirect immunofluorescence analysis (Fig. 2, left-hand column). PB1, PA, and NP proteins were observed in the nuclei and cytoplasm (Fig. 2).

Replication of NS-CAT RNAs in clone 64 cells. The chimeric NS-CATv RNA (vRNA sense) was transfected into both clone 64 and clone 76 cells that were incubated with dexamethasone. Total cellular RNAs were isolated 6 h after transfection, and the level of NS-CAT cRNA (cRNA sense) was analyzed by Northern hybridization. As shown in Fig. 3A, cRNAs were detected at almost the same level in clones 64 and 76. Next, the chimeric NS-CATc RNA (cRNA sense) was transfected into both cell lines and NS-CAT vRNA synthesis was examined. Northern hybridization revealed that vRNA was also synthesized from the cRNA at the same level in both cell lines (Fig. 3B). Furthermore, to confirm cRNA and vRNA syntheses from vRNA and cRNA, respectively, RT-PCR and RNase protection assays using clone 64 cells transfected with NS-CAT RNAs were performed. RT-PCR using oligonucleotides SN24

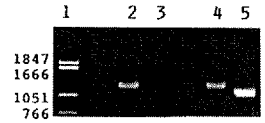

FIG. 1. Detection of PB2 and PB1 mRNAs by RT-PCR. Total cellular RNAs were prepared from clones 64 and 76 incubated with 10^{-6} M dexamethasone for 6 h. Twenty micrograms of total cellular RNAs (clone 64 cells [lanes 2 and 3] and clone 76 cells [lanes 4 and 5]) were amplified by RT-PCR using PB2-specific primers (PB2-SN3 and PB2-SN1 [lanes 2 and 4]) or PB1-specific primers (PB1-SN5 and PB1-SN4 [lanes 3 and 5]). RT-PCR conditions were as described in Materials and Methods. Lane 1, DNA size markers (sizes are indicated in base pairs on the left).

(to detect cRNA) and SN25 (to detect vRNA) and RNase protection assays using NS-CATv RNA (to detect cRNA) and SN-CATc RNA (to detect vRNA) revealed that the cRNA was synthesized from vRNA and vRNA was synthesized from cRNA in clone 64 cells (data not shown). These results indicated that exogenous viral RNA can be replicated in clone 64, as it is in clone 76.

The nature of poly(A)$^+$ RNA in NS-CATv RNA-transfected clone 64 cells. Since it is believed that the PB2 subunit is involved in generation of capped primers for viral mRNA synthesis (20), the nature of plus-stranded RNA in clone 64 cells transfected with the NS-CATv RNA was analyzed in detail. Total cellular RNAs were isolated from clones 64 and 76 6 h after transfection with the NS-CATv RNA and were then fractionated into poly(A)$^+$ RNAs with oligo(dT)$_{30}$-Latex. Poly(A)$^+$ CAT RNAs were detected in both cell lines (Fig. 3C). To negate the possibility that a little cRNA was present in the poly(A)$^+$ RNA fraction, Northern hybridization using the SN24 oligonucleotide probe was performed. No cRNA was detectable in the poly(A)$^+$ RNA fractions prepared from clones 64 and 76 (data not shown). These results indicated that clone 64 cells can synthesize poly(A)$^+$ CAT RNA from exogenous vRNA, as can clone 76. However, CAT activity was not detected in clone 64 cells transfected with NS-CATv RNA (Fig. 4, lanes 6 to 8). In separate experiments, no faint bands were evident in the NS-CATv-transfected clone 64 cells and faint bands such as those in Fig. 4, lanes 6 and 7, were sometimes detected in the negative control (data not shown). Thus, the faint bands in lanes 6 and 7 seem to be artifacts. In the NS-CATv RNA-transfected clone 76 cells, CAT activity was detected 21 h after transfection, reaching a maximum level after 27 h and slightly decreasing after 33 h (Fig. 4, lanes 2 to 4). These results indicated that the poly(A)$^+$ CAT RNA in clone 64 cells is not translatable.

Since CAT activity was undetectable in the NS-CATv RNA-transfected clone 64 cells despite the sufficient production of poly(A)$^+$ CAT RNA, we attempted to translate in vitro poly(A)$^+$ RNA prepared from the NS-CATv RNA-transfected clone 64 cells. Poly(A)$^+$ RNAs prepared from clone 64 and

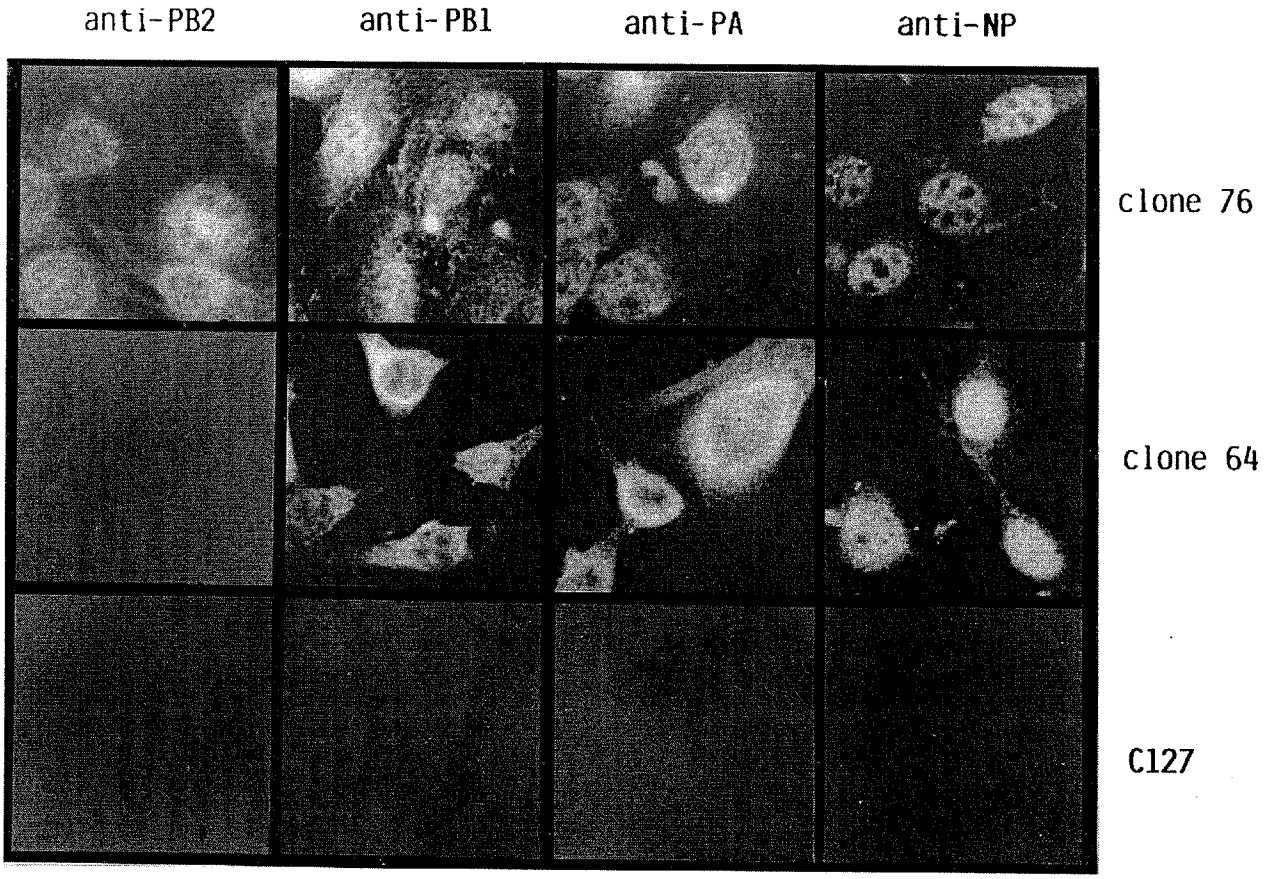

FIG. 2. Expression of RNA polymerase subunit genes and NP gene in clone 64, clone 76, and parental C127 cells. Cells cultured in the presence of 10^{-6} M dexamethasone for 17 h were incubated with anti-PB2, anti-PB1, anti-PA, or anti-NP antibodies (as indicated above the columns) for indirect immunofluorescence. The cell lines are indicated at the right of the rows.

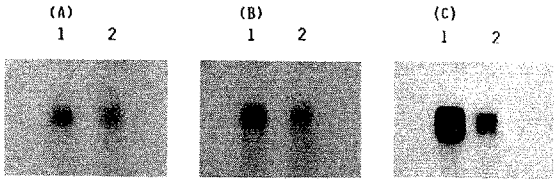

FIG. 3. Northern blots of NS-CAT RNAs in clones 64 and 76. Clones 64 and 76 incubated with 10^{-6} M dexamethasone were transfected with NS-CATv (antisense orientation) (A and C) or NS-CATc (sense orientation) (B) RNA. Twenty micrograms of total cellular RNAs (A and B) or 1.3 μg of poly(A)$^+$ RNA (C) prepared 6 h after transfection was resolved by electrophoresis on 1% formaldehyde-agarose gels. The cRNA (A), vRNA (B), and poly(A)$^+$ CAT RNA (C) transcripts were analyzed by using ^{32}P-labeled SN24 (complementary to the 3′ end of cRNA), SN25 (complementary to the 3′ end of vRNA), and CAT cDNA probes, respectively, by Northern hybridization. Lanes 1, clone 64 cells; lanes 2, clone 76 cells.

clone 76 cells were incubated in rabbit reticulocyte lysate containing [^{35}S]methionine for 60 min, and the labeled proteins were immunoprecipitated with rabbit anti-CAT antibody and analyzed by SDS-polyacrylamide gel electrophoresis. We detected a discrete band with an apparent molecular weight of 30,000 to 32,000 in immunoprecipitates from clone 76 cells, which corresponded to the size of the fusion CAT protein with an extra 19 amino acids of the N-terminal region of NS1 protein (40) (Fig. 5A, lane 1). Another band at the position corresponding to a molecular weight of 25,000 to 26,000 seemed to be the authentic CAT protein initiated from the initiation codon of the CAT gene. However, no detectable band was observed with poly(A)$^+$ RNA from clone 64 cells (lane 2) or from the parental C127 line (lane 3). These results also indicated that the poly(A)$^+$ CAT RNA in clone 64 cells cannot be translated.

Capping of poly(A)$^+$ RNA from NS-CATv RNA-transfected clone 64 cells. One possible explanation is that the poly(A)$^+$ CAT RNA in clone 64 cells does not contain a cap structure at the 5′ end. To test this possibility, the poly(A)$^+$ RNAs prepared from clones 64 and 76 were incubated with purified yeast mRNA capping enzyme in the presence of [α-^{32}P]GTP. If the cap structure is absent in the poly(A)$^+$ CAT RNA and if the RNA molecules carry either pppN or ppN at the 5′ termini, the cap structure can be added in vitro to these termini, as the purified yeast mRNA capping enzyme carries both RNA 5′-triphosphatase and RNA guanylyltransferase activities (15). After the capping reaction, the products were analyzed by electrophoresis on a 3% polyacrylamide gel containing 7 M urea. A discrete ^{32}P-labeled band was identified from clone 64 cells at a position corresponding to that for a sequence of 890

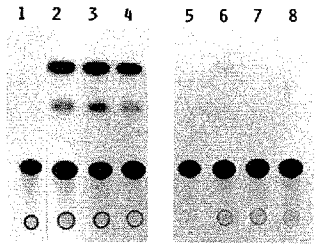

FIG. 4. Expression of NS-CATv RNA transfected in clones 64 and 76. NS-CATv RNA was transfected into dexamethasone-treated clones 76 (lanes 2 to 4) and 64 (lanes 6 to 8). The cells were harvested at 21 (lanes 2 and 6), 27 (lanes 3 and 7), or 33 (lanes 4 and 8) h after transfection. The CAT assay was performed with crude cell extracts. Lanes 1 and 5, mock-transfected clone 76 and clone 64 cells, respectively.

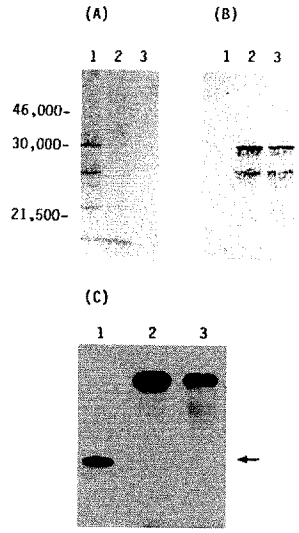

FIG. 5. In vitro translation of NS-CAT-specific poly(A)$^+$ RNA before (A) or after (B) in vitro capping. (A) Poly(A)$^+$ RNAs (0.2 μg) prepared from clone 76 (lane 1), clone 64 (lane 2), or C127 (lane 3) cells were translated in a rabbit reticulocyte lysate containing [^{35}S]methionine. (B) Poly(A)$^+$ RNAs from clone 64 (lane 2) or clone 76 (lane 3) were capped and methylated with vaccinia virus capping enzyme and were then translated in the rabbit reticulocyte lysate containing [^{35}S]methionine. The translation products were immunoprecipitated with anti-CAT antibody and were loaded onto a 7.5 to 15% gradient SDS-polyacrylamide gel. Lane 1, uncapped clone 64 poly(A)$^+$ RNA. The positions of size markers are shown at the left. (C) In vitro capping of poly(A)$^+$ CAT RNA prepared from clone 64 cells. Poly(A)$^+$ RNAs prepared from clone 64 (lane 3) and clone 76 (lane 2) cells were incubated with yeast mRNA capping enzyme and [α-^{32}P]GTP prior to analysis by electrophoresis on a 3% polyacrylamide gel containing 7 M urea. Lane 1, RNA size marker of 890 nucleotides.

bases (Fig. 5C, lane 3). This size exactly corresponded to that of the poly(A)$^+$ CAT RNA. Other bands indicating sequences shorter than 890 bases (cRNA replication intermediates) were not evident. For poly(A)$^+$ RNA from clone 76 cells, a weaker band was detected at the same position (lane 2). The capping enzyme isolated from vaccinia virus also produced similar results (data not shown). These results suggested that the level of uncapped RNA is higher in poly(A)$^+$ CAT RNA from clone 64 cells than that from clone 76.

To confirm this conclusion, a cap structure was added in vitro to the 5′ end of poly(A)$^+$ CAT RNA prepared from clone 64 cells, and its translatability was examined in vitro. Poly(A)$^+$ RNA from clone 64 cells was incubated with the capping enzyme prepared from vaccinia virus, which contains mRNA (guanine-7-)methyltransferase activity in addition to RNA phosphatase and guanylyltransferase activities, in the presence of *S*-adenosylmethionine, because a methylated cap structure is required for efficient translation (26, 27). When capped and methylated poly(A)$^+$ RNA from clone 64 cells was translated in the reticulocyte lysate, a discrete band was detected at the same position as the fusion CAT protein observed with poly(A)$^+$ RNA from clone 76 cells (Fig. 5B). These results indicated that the poly(A)$^+$ CAT RNA from clone 64 cells lacks the cap structure but that it can be capped in vitro.

DISCUSSION

In clone 76 cells, all three polymerase subunit genes and the NP gene of influenza virus can be expressed in response to dexamethasone. Under induced conditions, this cell line can complement the growth of all three types of temperature-

sensitive polymerase mutants at nonpermissive temperatures (28). Furthermore, exogenous NS-CAT chimeric RNAs can be replicated and transcribed in response to dexamethasone (17, 18).

For transfection of negative-stranded viral RNA, Huang et al. (13) have designed an artificial vaccinia virus vector-driven replication system and de la Luna et al. (7) also have described an artificial simian virus 40 vector-driven replication system. In both systems, CAT activity was detected after transfection of negative-sense CAT RNA in cells that simultaneously expressed all three polymerase subunit proteins (PB1, PB2, and PA) and NP protein. The activity was, however, not observed in cells that did not express any of these proteins.

We established the clone 64 cell line by transfection into mouse C127 cells with expression plasmids pBMSA-PB1, pBMSA-PB2, pBMSA-PA, and pBMSA-NP, in which the PB1 and PA subunit genes and the NP gene can be expressed in response to dexamethasone (28). The induced levels of the PB1, PA, and NP genes in clone 64 cells are almost the same as those in clone 76 cells (28). Since they lack PB2 protein, clone 64 cells cannot complement the growth of three polymerase temperature-sensitive mutants at nonpermissive temperatures (28). In this study, we examined the replication and transcription of an exogenous chimeric RNA of influenza virus in the clone 64 cells. Highly sensitive DNA detection methods failed to reveal the PB2 gene in clone 64 cells (28). We tried to detect the PB2 mRNA, the PB2 gene, and PB2 protein in clone 64 cells by RT-PCR, PCR, and indirect immunofluorescence analysis, respectively, but all failed. Thus, we concluded that the PB2 subunit gene is absent from clone 64 cells.

Indirect immunofluorescence showed that the proteins expressed in clone 76 (PB2, PB1, PA, and NP) and in clone 64 (PB1, PA, and NP) were localized not only in the nuclei but also in the cytoplasm (Fig. 2), even though there are intrinsic nuclear localization signals in PB1 (1, 29, 34), PB2 (16, 25, 34), PA (30, 34), and NP proteins (6). The different profiles of polymerase subunit localization and NP protein localization of the cells may reflect a difference in the nuclear transport systems.

Both cRNA synthesis from vRNA and that of vRNA from cRNA were detected in clone 64 cells incubated with dexamethasone, as they were in clone 76 (Fig. 3A and B). These results indicated that clone 64 carries an apparatus that can replicate the exogenous viral RNA and that the PB1 and PA subunit genes as well as the NP gene are sufficient for the construction of this machinery. In parallel, we examined the transcription level of exogenous NS-CATv RNA. Northern hybridization revealed that poly(A)$^+$ CAT RNA was synthesized in clone 64 cells at almost the same level as that in clone 76. Although Fig. 3C shows a higher level of poly(A)$^+$ CAT RNA in clone 64 than in clone 76, the higher level of poly(A)$^+$ CAT RNA in clone 64 was not constant. However, the poly(A)$^+$ CAT RNA in clone 64 cells could not be translated (Fig. 4 and 5), because the poly(A)$^+$ CAT RNA does not have the cap structure at the 5′ terminus. In fact, it was capped in vitro by capping enzymes isolated from yeast cells and vaccinia virus (Fig. 5C). Furthermore, after the in vitro capping with the enzyme prepared from vaccinia virus, which contains mRNA (guanine-7-)methyltransferase activity in addition to RNA phosphatase and guanylyltransferase activities, this poly(A)$^+$ CAT RNA was translated in vitro into CAT protein (Fig. 5B). Furthermore, when clone 64 cells were infected with the PB2-expressing vaccinia recombinant virus, PB2-VAC (34), CAT activity was detected after an incubation with dexamethasone (data not shown). These results indicated that PB1, PA, and NP are sufficient for the synthesis of poly(A)$^+$ RNA but that

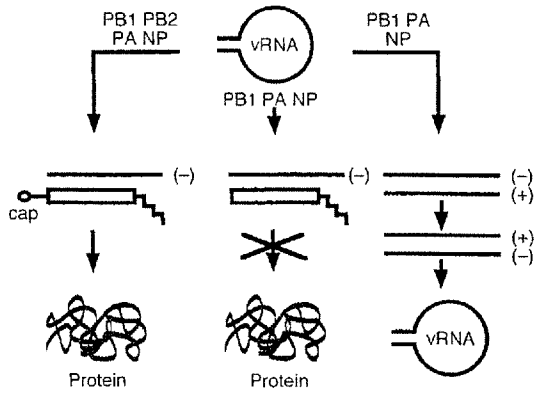

FIG. 6. Roles of RNA polymerase subunits and NP in replication and transcription of the influenza virus genome. PB1 and PA subunits of RNA polymerase and NP are sufficient for replication of the influenza virus genome (right-hand column) and transcription to yield uncapped poly(A)$^-$ RNA (middle column). The PB2 subunit is required for transcription to form capped poly(A)$^+$ RNA (left-hand column). −, vRNA; +, cRNA.

PB2 is required for cap recognition. Our results are consistent with observations that PB2 recognizes and binds to the cap structure of the cellular mRNA (for a review, see reference 20). Figure 6 shows a schematic illustration of the roles of RNA polymerase subunits in the replication and transcription of the influenza virus genome.

The role(s) of each subunit of RNA polymerase has been studied in vitro by using reconstituted RNA polymerase (19, 31, 35). To analyze the in vivo functions of each RNA polymerase subunit, however, our in vivo replication-transcription system using established cell lines and the model templates may be useful.

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